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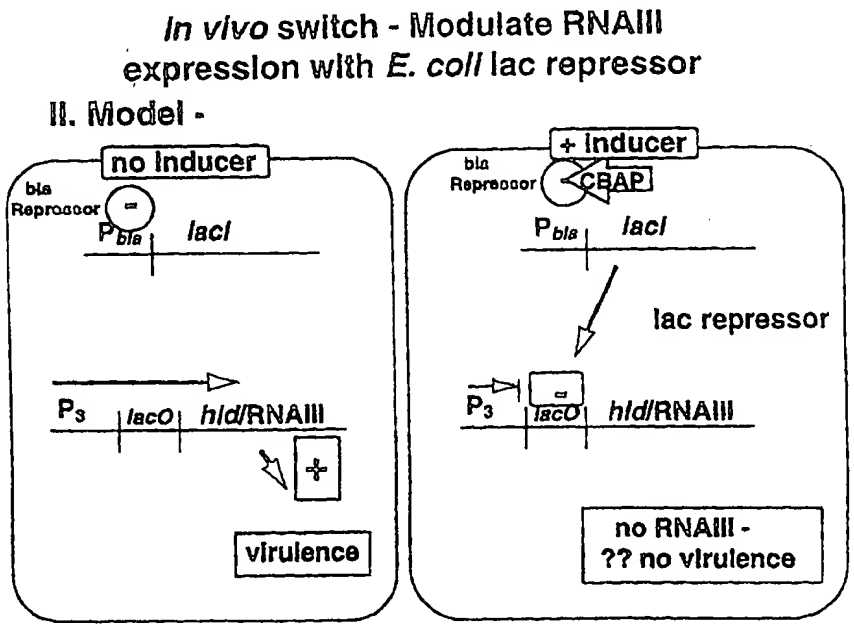

(57) Abstract

Methods for evaluating microbial genes as targets for compounds which inhibit growth of a microbe or alter the pathogenesis of a microbe, and for evaluating the expected therapeutic effect of compounds which inhibit a reaction of a microbial cell which is related to the expression of a specific gene. The methods utilize recombinant microbes which contain DNA construct or alterations, such that the level of activity of the products of coding regions associated with those constructs of alterations can be controlled by contacting the microbe with a specific small molecule or other specific artificial environmental change. Such recombinant microbes can be used in infection models to evaluate the effect of artificially changing the level of activity of a product of a gene.

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DESCRIPTION

Methods for Evaluation of Antimicrobial Targets

Background of the Invention

This invention relates to the fields of antimicrobial therapy and methods for identifying and evaluating targets for antimicrobial therapy.

The process by which bacterial pathogens and other microorganisms cause disease involves the overt replication of the microbe within the host, and/or the production of both cellular and extracellular factors which enhance the pathogenicity of the microbe. To be an effective pathogen the invading microbe must establish itself in the host, creating an environment in which it can avoid destruction by the host's immune system. To establish itself, the microbe requires a variety of molecules which provide functions such as attachment to host tissue, penetration of anatomic barriers, disruption or avoidance of humoral factors, and avoidance or inactivation of phagocytic cells. Such molecules include colonization factors, such as adhesins, and certain proteases, endotoxins, and exotoxins. These molecules help to secure the microbe to favorable host tissues and to alter the local environment to allow initiation and maintenance of infection. This process is often very specific for a host/pathogen pair, and is essential for the subsequent events in the pathogenic process.

Once established, the pathogen may produce a variety of virulence factors or toxins. These microbial products are usually genetically regulated and the microbe often expresses these products only while within the host. These virulence factors include a variety of toxins and other exoproducts important for the creation of and maintenance of the ecological niche within which the microbe resides. For example, for the enteric pathogen, *Shigella*, clinical isolates produce pili to adhere to

mucosal surfaces and toxins which enhance invasion of mucosal cells. In *Staphylococcus*, a large number of pathogenesis-related exoproducts have been identified, including Staphylococcal lysozyme, exfoliative toxins A and B, pyrogenic exotoxin, coagulase, hemolysins a-d, and collagenase. In other pathogenic species other virulence factors have been identified, but numerous virulence factors remain to have their functions identified or fully appreciated. All important pathogens have similar capabilities. These capabilities vary among species and depend on the environment in which the pathogen is capable of prospering, as well as the genetic capabilities of the microbes.

As was suggested above, virulence factors include many molecules besides toxins and should be regarded as including any microbial products which enhance the capability of the microbe to create disease. So, for example, capsular polysaccharides produced by the microbe act as anti-phagocytic defenses to prevent the hosts' immune system from eliminating the microbe. There are additional virulence factors which enhance the capability of the invading microbe by avoiding or disrupting humoral (antibody-mediated) immunity. There are also virulence factors which give the invading microbe properties which overcome the hosts' anatomic barriers. All such microbial properties which help to create or maintain a microbe's ability to cause disease are possible targets for novel antimicrobial agents.

The bacteria are not alone in the microbial world in their ability to cause disease. In addition to numerous viruses, a number of lower eukaryotes, fungi and yeast are being recognized as increasingly important pathogens. In such pathogenic fungi and yeast, like in bacteria, the organisms possess specific features which enhance the ability to cause disease. Parasites also have a wide variety of described and yet to be described virulence factors.

None of the above material is admitted to be prior art to the pending claims, but is provided only to aid the understanding of the reader.

Summary of the Invention

This invention provides methods for evaluating specific microbial genes and the encoded products as targets for antimicrobial therapy prior to identification of a specific inhibitor(s) or enhancer(s) of the gene or gene product. These methods are useful for evaluating such genes and gene products even when information about the function of the encoded products is incomplete. For example, such evaluations can be performed both when the reaction pathways involving a gene product are known to some extent, but before the involvement of that product in an infection process is fully appreciated, and when the specific reaction pathways in which the gene product is involved are known. Since, the methods utilize expressed coding sequences, evaluations can be performed even before the specific gene product has been characterized. Thus, these methods provide an efficient approach to the identification and evaluation of novel therapeutic targets.

In general, these methods for evaluating antimicrobial targets are based on artificial control of the level of activity of a gene product. As described herein, such control may be achieved in different ways, but generally involve artificially changing the environment of a microbe (such as by contacting the microbe with a small molecule) which causes a change in the level of activity (or function) of a gene product. The change in activity can be accomplished in a variety of ways, among which are altering the transcription rate, altering mRNA processing (for cells and genes for which this is necessary), transport, or degradation, altering the translation rate, and altering the stability of the gene product. (Alberts et al., MOLECULAR BIOLOGY OF THE

CELL, 3rd ed., p.403.) Any of these processes can be used to alter the level of activity of the product of a gene, and so could be utilized in the methods of this invention.

Along with the identification of novel targets, these methods allow assessment of time-dependent inhibition of a target gene product, *i.e.*, these methods allow assessment of the consequences of inhibiting the target gene product at various times after infection is initiated and/or of various dosing regimes (*e.g.*, different schedules of multiple dosing). Such information has clinical relevance for the possible therapeutic administration of any antimicrobial agent which affects that target (such as the possible efficacy), and are important properties to characterize for each potential target. The differing temporal importance of various gene products generally is primarily due to infection-related changes in the infection host environment (includes both global host environment and the localized site of infection) which result from the infection.

The use of a variety of infection models also allows evaluation of the effects of different infection sites in relation to varying inhibition schedules. The site of infection effects are also generally related to the infection-related changes in the host environment and also have clinical relevance. Such determination of infection site differences can also be combined with temporal evaluations as described above.

In addition, by use of various animal (or other whole organism) or cell-based infection models, the expected therapeutic benefit of an inhibitor of the gene, gene product, or reaction pathway, which may later be found, can be estimated prior to any extensive search for such an inhibitor. This includes an assessment of the tissue-specific effects of the inhibition of a specific target by comparative evaluation of the effects observed in the different infection models. In addition, certain models can provide information on the tissue-specificity of the

infecting microbe, as well as of the pattern of clearance of the microbe from the infected host.

Still further, these methods provide a standard for the comparison of the effects of the *in vivo* administration of a compound which inhibits a specific target to affect a microbial infection. These methods allow determination of the effects of complete loss of function of the product of a test gene at one or at various times after an infection is initiated. The effects of complete loss of function can then be compared with the effects of administration of an inhibitor at similar time points after an inhibitor has been found. The capability to completely inhibit the activity of a gene product therefore provides a baseline for evaluating the effects of an inhibitor.

Thus, in a first aspect, this invention provides a method for evaluating a putative pathogenesis gene or essential gene as a target for antimicrobial treatment. This method involves infecting a mammal with a microbe containing an artificially-created genetic alteration, where the genetic alteration causes a change in the level of activity of a product of the coding sequence of that putative pathogenesis gene or essential gene in the microbe, in response to an artificial change in the environment of the microbe. When the environment is changed in that way, the level of activity of a product of the coding sequence is changed, and the method involves determining whether the state of the infection or the physiological condition of the mammal is altered in response to the change in the level of activity of the product of the coding sequence. The putative pathogenesis gene or essential gene is a target if the state of the infection or the physiological condition of the mammal is altered in response to the change in the level of activity of a product of the coding sequence. This method includes the use of microbes with more than one test gene. In one arrangement, two or more coding sequences could be linked

together which are naturally linked (such as an operon), or two or more coding sequences could be transcriptionally linked by artificial manipulations known. However, the coding sequences can also be separate. The techniques for manipulating coding sequences and other DNA or RNA sequences are well-known in the relevant scientific communities.

In preferred embodiments, the artificial change of environment is achieved by contacting the microbe with a switching compound. As is described herein, such a switching compound is preferably a small molecule with well-characterized pharmacodynamics in the infection host. An understanding of the pharmacodynamics of the switching compound enables the selection of a switching compound appropriate for a specific pathogenic microbe or infection model, as well as modeling the therapeutic consequences of the use of an inhibitor of a specific activity which has similar pharmacodynamics (including tissue distribution and clearing characteristics). In general, the choice of a switching compound depends on a number of factors, including, *e.g.*, sufficient distribution of the compound to the infected tissue and sufficiently long half-life of the compound in the infected tissue to achieve effective control of the level of activity of the test gene. However, the choice of a switching compound and the choice of switch design are related; selection of one of these will often limit the possible selections of the other. It should be clear that a switching compound as described herein, can, in various switch designs, be any of a variety of biologically active compounds. Some such compounds can be referred to using other biological terms, such as "modulators", "enhancers", "inducers", "mediators", and "regulators." Therefore, this invention is not limited by the specific control molecule used, nor by the specific switch design chosen to control the level of activity of a product of a test gene.

Also in certain embodiments, the methods use a microbe which has more than one artificially-created genetic alteration, each of which enables a change in the level of activity of a product of a test gene. At least two of the genetic alterations enable changes in the level of activity of the test gene in response to different switching compounds. One way of constructing such a system is to have expression of a test gene blocked by binding of a repressor molecule. Expression of the repressor molecule can then be turned on by the presence of any of several switching compounds (which then stop expression of the test gene). Such a microbe thus allows convenient comparison of the effects of altering the level of activity of the test gene by the use of compounds with a range of pharmacodynamic properties. (See Fig. 8 for schematic depiction of an example using two different switching compounds.)

Similarly, in certain embodiments, the method uses a microbe which contains more than one artificially-created genetic alteration, and more than one test gene. The levels of activity of at least two of the test genes can be changed in response to different switching compounds. Such microbes allow the convenient evaluation and comparison of the effects of changing the levels of activity of different gene products in the same microbial and infection environments. Such microbes can also be used to evaluate the effects of more than one test gene concurrently.

The term, "artificially-created genetic alteration", refers to a change in the DNA of an organism through the actions of a person, but does not refer to changes occurring through natural processes of genetic exchange, such as conjugation. Such artificially-created genetic alterations can be of many different types. Some of those types include single nucleotide changes, deletion of a DNA sequence(s), insertion of a DNA sequence(s), or combinations of the preceding changes. Further, the

changes may be to the DNA of the chromosome(s) or to DNA which is extra-chromosomal. Also, the changes can be located in coding sequences or in other sequences such as regulatory sequences, including binding sites for components related to transcription or translation. Such artificially-created genetic alterations can be transmitted through generations, and each transmission results in a new organism. Such new organisms are also said to have artificially-created genetic alterations.

As used herein, the term "extra-chromosomal" refers to DNA which is part of a DNA molecule which can be replicated within a cell, but which is not part of the chromosome of the cell. Examples of such non-chromosomal, replicatable DNA molecules include a large number of plasmids, but can also include other categories of DNA molecules, *e.g.*, cosmids and yeast artificial chromosomes (YACs). Since such molecules can be replicated in the cell, copies can be passed to succeeding generations of the cell, maintaining the presence of the extra-chromosomal DNA.

The phrase, "level of activity of a product of the coding sequence", refers to the level of physiologically relevant biological function of the product of the coding sequence being tested. For example, for an enzyme, this refers to the level of activity of all of the molecules of that type present. The level of activity of a product can be altered in a variety of ways, including altering the rate of transcription to form RNA, altering the rate of translation of a polypeptide product from a mRNA, altering the transport of a product to a site of action, and altering the stability of the product to change the active lifetime of the product. Any of these methods can be used to alter the level of activity of the product of a coding sequence. In the context of these methods, a "change" in the level of activity of the product of a coding sequence is determined by comparison with the level of the activity in the absence of the artificial change in the

environment. That comparison level of activity may be the same or different than the level of the same activity in a corresponding wild-type organism.

The term, "artificial change in the environment", refers to a change in the environment of a microbe which is directly caused by the actions of a person. In particular embodiments, such changes specifically include changing the concentration of a small molecule (such as by adding a quantity of that compound) in the vicinity of a microbe. However, the term can also refer to other changes such as exposure to light (which may be of a specific wavelength), contact with a specific metal ion(s), or a change in temperature.

In a related aspect, this invention provides a method for evaluating a specific putative pathogenesis gene or essential gene, or the gene product of such a gene, or the reaction pathway in which the gene product is an important element, as a target for antimicrobial treatment. In this method, recombinant microbes which contain one or more DNA constructs are used to infect a mammal. At least one of the DNA constructs contains a coding region of a putative pathogenesis gene or essential gene, such that the expression of that gene can be controlled by contacting the microbes with a switching compound. The control, *i.e.* the ability to switch, or change, the expression state, mimics the effect of an antimicrobial agent, for example the effect of an inhibitor of the product of the switched coding region. After switching the expression state of the putative pathogenesis or essential gene, the method involves determining whether the state of the infection or the physiological condition of the animal was altered in response to the switch of the expression state of the gene. The gene is a target if the state of the infection or the physiological condition of the animal is altered in response to the switch of the expression state of the gene.

Thus, the *in vivo* effects (meaning effects occurring with the microbe in the infection host environment) which result from changes in the function of the infecting microbe due to the administration of such an inhibitor can be determined prior to the discovery of any such inhibitor. This information provides a measure of the potential effectiveness of such an inhibitor in treating microbial infections, which provides valuable guidance on whether to pursue discovery experiments searching for such an inhibitor. One useful and measure is the "cidalness" of the test gene, *i.e.*, the degree to which loss of the function of the test gene kills the infecting microbe.

In addition to providing an evaluation of the gene and its corresponding product and reaction pathways as potential antimicrobial targets, this method provides additional information on the appropriateness of the pathogenesis gene or essential gene as a practical antimicrobial target. Such information is provided by following the initial evaluation of whether the microbial infection or the health of the infected animal is altered in response to the switch of the expression state of the pathogenesis gene or essential gene, with an evaluation of the extent, nature, and consequences of the alteration of the infection or the health of the host.

The term "method for evaluating" means a method for determining whether a property or characteristic or effect is present. This may, but does not necessarily, include a measure of the size, level, or intensity of such characteristic property or effect.

A "pathogenesis gene" is a gene whose function is critical at least at some point in the pathogenesis process, *e.g.*, in the establishment, maintenance, *in vivo* survival, or progression of an infection, or in the maintenance of a latent or quiescent state of an infection in an organism, *e.g.*, a mammal or other animal or a plant. Therefore, as examples, this term includes genes for products which allow a microbe to adhere, colonize, and

survive in the host environment. These include genes for products such as adhesion factors, which allow the microbe to attach to a surface of the host organism, as well as genes for products which alter the microbial environment at the infection site and for products which reduce the ability of the host's immune system to eliminate the infection. In the context of this method for evaluating a gene as a target, this implies evaluation not only of the gene itself, but of the transcribed RNA, the expression product, and of a reaction pathway or pathways in which that expression is an important part. Consequently, the term does not imply that an inhibitor necessarily acts at the level of the DNA or transcription process; in many cases an inhibitor would be expected to act at the level of a reaction directly involving the expression product of the gene (*e.g.*, inhibition of an enzyme).

A "putative pathogenesis gene" is a gene which is being evaluated as a target for antimicrobial therapy. This does not imply that it is known that the gene is a pathogenesis gene as described above. For use in this method, a putative pathogenesis gene has preferably, but not necessarily, been chosen on the basis of some prior information about the gene or a relationship of the gene with other genes believed to be pathogenesis genes. Examples of such other types of information are provided below in the Description of the Preferred Embodiments.

The term "essential gene" refers to a gene whose function is essential to the effective growth of the microbe in a particular growth condition(s), *e.g.*, in *in vitro* culture in a defined medium. For identification as a possible antimicrobial target, a gene identified as essential *in vitro* is also essential *in vivo*, that is, in an infection, such as of a mammal, and is preferably a gene whose function is essential to survival of the microbe *in vivo*. Similar to the description of pathogenesis genes above, the term "essential gene" refers

not only to the DNA coding region, but also to the RNA transcript and the expression product. It also refers to the reaction pathways of which the expression product is an important part. Stating that the product is an important part of a reaction pathway means that the absence or essentially complete inactivation of that product substantially changes the level of a functionally relevant activity in the microbe. This implies that there are not alternative products or pathways to return the relevant activity to a functionally equivalent level.

A "putative essential gene" is a gene which is being evaluated in this method in a manner suitable for essential genes. These genes may, for example, be selected for testing on the basis of prior information about their essential status, or on the basis of other information, *e.g.*, homology information for the DNA coding sequence with known essential genes from other related microbes. Prior information about the status of a gene as essential can, for example, be provided by the isolation of temperature sensitive mutants, mutated in a gene corresponding to the test gene.

In the context of the DNA constructs of the claims of this invention, the term "gene" refers to a DNA sequence which is the same as all or a portion of the coding sequence of a gene from a microbial genome. However, same does not mean identical. Thus, the sequences may differ by changing a small percentage of bases, by deleting a small percentage of bases, by adding a small percentage of bases, or by a combination of such differences. Such differences are preferably less than 5 or 10% of the full sequence, but in some cases may be higher, *e.g.*, 20% or 30%. It should be recognized that the biological activity of interest may depend on the presence of only a portion of the natural gene product, so the product expressed by the gene on the DNA construct should have essentially the same biological function as the product of the native gene. In addition, the biological activity for a fixed

concentration of gene product is preferably approximately the same for the product of the DNA construct as for the product of the native gene. However, if the natural gene product possesses multiple biological functions, the product of the test gene need not possess all of those activities.

As was suggested above, evaluating a gene as a target means determining whether an alteration of the expression of the gene alters the status or course of the microbial infection, or the health of the infected organism (*e.g.*, a mammal). Therefore, altering the expression of the gene can mimic the action of antimicrobial agents which may act at the level of the DNA coding sequence itself, but this is not essential for identifying the gene as a target. It can equally mimic antimicrobial agents which act at the level of the RNA transcript or directly on the expression product, or which interfere in some manner with the reaction pathway of which the expression product of the gene is an important part. Therefore, in this method, a gene is a "target" if an alteration in the expression of that gene results in a change in the status or course of the microbial infection, or in the health of the infected animal.

The term "mammal" has it usual biological meaning as referring to warm-blooded animals which nurse their young. This includes, *e.g.*, mice, rats, rabbits, dogs, cats, swine, cattle, and humans.

The term "antimicrobial treatment", as used herein, refers to the administration of a compound to interfere with the growth, viability or effects of the presence of a population of a microbe. To illustrate, a compound administered as an antimicrobial treatment may kill the microbe, or, as another possibility, the compound may merely enhance the capability of an infected host animal to eliminate the microbial infection.

The term "infection" refers to the presence of a population of a microbe in an animal where the presence of

that population is to some degree damaging to the host animal. This effect may be due, for example, to the presence of excess numbers of the microbe, or to the presence of a highly virulent strain or sub-population of a strain of the microbe, or may be due to the presence of a population of a microbe which is not normally present in the animal or which is present in an abnormal location in the animal.

The term "microbe" includes the usual biological meaning. Therefore, the term includes, *e.g.*, bacteria, protozoa, fungi, and yeast, as well as viruses. A specific example of a bacterium which is described and utilized in the description of the preferred embodiment and the examples is *Staphylococcus aureus*. It should be recognized that species of many microbes, such as bacteria, can exist as a variety of strains which may have genetic differences which are significant for pathogenesis. The term "recombinant microbe" similarly has its usual molecular biology meaning. The term refers to a microbe into which has been inserted, through the actions of a person, a DNA sequence or construct which was not previously found in that microbe, or which has been inserted at a different location within the cell or chromosome of that microbe. Such a term does not include natural genetic exchange, such as conjugation between naturally occurring organisms. Thus, for example, a recombinant bacterium could have a DNA sequence inserted which was obtained from a different bacterial species. Likewise, a recombinant bacterium may contain an inserted DNA sequence which is an altered form of a sequence normally found in that bacterium. Another example of a possible insert in a recombinant bacterium is the insertion of a DNA sequence which is normally found in the bacterium, but which is in a construct different than the DNA structure in which the sequence was previously found in the bacterium. An example could be a sequence on a plasmid, when the sequence was previously chromosomally

located. In general then, a recombinant microbe is a microbe into which a DNA sequence has been inserted by artificial genetic manipulations. For most purposes it is preferable that the inserted DNA sequence should be a stable insertion, meaning that the sequence should be replicated and transmitted to progeny microbes as growth occurs.

In the context of recombinant microbes, a "DNA construct" describes a DNA chain which contains a segment which has been removed from its normal microbial environment. Thus, for example, a DNA construct could consist of a coding sequence with its normally associated regulatory sequences, which has been isolated from its normal microbial setting, but a DNA construct can also include a DNA coding region which has been inserted in a microbe in a plasmid in which it was not previously found, or a DNA regulatory region which has been inserted into a microbial chromosome. A variety of other such DNA constructs are well known to, and used by, those skilled in the art.

In the context of the coding region of a gene, "expression state" indicates the status of the production of the encoded product of that coding sequence. Thus, for most genes, the expression state refers to the status of the translation of an RNA transcript to form a polypeptide product, but can also refer to the status of the transcriptional product when that transcriptional product has the relevant biological activity. An example of such an active transcriptional product is RNA III, which is related to the *agr* locus of *Staphylococcus aureus*. At the extremes, "expression state" indicates whether detectable gene product is being produced or not. If detectable gene product is being produced, the expression state is "on", if not, the expression state is "off". In addition, however, expression state can refer to the level of production of a gene product. Therefore, it can refer to

whether the product is being produced at a low level ("off") or a high level ("on").

For a related term in the context of the expression state of a gene, the term "switched" means that the level of production of the gene product of the coding sequence has been detectably altered, *i.e.*, the expression level has been changed. Thus, for example, the expression state of a coding sequence has been switched if the level of expression has been changed from a low level to a high level, where, in some instances, the low level may be undetectable and may therefore be referred to as no production. Similarly, a change in the expression level may be in the opposite direction, from a high level of production to a low level. As indicated above, a low level of production may be undetectable. As suggested above, the expression state of a gene may be switched by alteration at the transcriptional level or the translational level. As described above, the level of activity of the product of a gene can be changed in other ways, *e.g.*, by altering the stability of the product. Therefore, more generally, "switched" or "changed" means that the level of activity of a product of a coding sequence of a putative pathogenesis gene or essential gene is either higher or lower than before being switched. Constructs and methods which allow switching or changing *in vivo* are said herein to comprise an "*in vivo* switch".

The term "contacting", as used herein, indicates that a concentration of a compound or other material (*e.g.*, an ion such as mercury or iron ions) has been placed in the immediate cellular environment of the microbe. Such a compound may be, but is not necessarily, then taken up into the interior of the microbial cell. Therefore, the term "contacting" merely means that the exterior of the microbial cell has been exposed to the compound. For viruses, the term may mean that the compound has penetrated to the interior of the host cell, or merely

mean that the exterior of the host cell has been exposed to the compound.

In the context of the methods of this invention, a "switching compound" is one which alters (switches) the level of function of a product of a coding sequence, such as by altering the amount of functional expressed product present. In certain preferred embodiments the switching compound alters the expression level of a coding sequence of a test gene when the cell or the expression system containing that coding sequence is contacted with that switching compound. A well known example of such a compound which operates in a number of bacterial species is tetracycline. In certain bacteria, exposure to a low level (sub-growth inhibitory level) of tetracycline induces a much-elevated level of expression of a gene from a resistance-related promoter. A switching compound may cause an increase or a decrease in the level of activity of functional expressed product, *e.g.*, it may cause an increase in the expression level of a coding sequence or it may cause a decrease in the expression level of a coding sequence.

It is preferable that a switching compound be a small molecule which has well-characterized pharmacological properties. This is useful for several reasons. One such reason is that different compounds will distribute differently, and have different tissue clearance rates. This means that the effective concentration of a switching compound at different specific infection sites can vary. (Or conversely, that the effective concentration of different switching compounds at a specific infection site can vary even if the average concentration of the different compounds in the bodies of the infection hosts is the same.) This further means that the temporal behavior of various switching compounds will differ, with accompanying differences in the control of the level of activity of a gene. Therefore, a switching compound can be selected which is appropriate for evaluating a specific

test gene in a specific infection model based on suitable pharmacodynamic properties. Also, selection of a switching compound with specific known temporal and distributional characteristics *in vivo*, for use in evaluating a test gene, provides additional information on the likely therapeutic effects of inhibiting the test gene with an inhibitor which has similar characteristics, in comparison with the use of a switching compound (or other method of altering the level of activity of the product of a test gene) with unknown pharmacodynamics. Such compounds are known, for example, among the inactive (or low activity) analogs of known antibiotics. For these compounds, the pharmacodynamics of the class of compounds has been previously investigated, but the inactive analog has the advantage that it does not have significant antimicrobial activity to complicate the evaluation of potential targets (*e.g.*, the β-lactam, CBAP). Similarly, antibiotics to which the infecting microbe is resistant can be used to evaluate test genes unrelated to that resistance.

In addition, the use of different switching compounds which have varying access to different tissues can provide additional information on the suitability of a target in relation to dissemination of the infection, and of microbial load in the different tissues. In certain cases, a range of switching compounds can be used, each of which will switch the level of activity of the product of one coding region (in conjunction with appropriate regulatory components) in order to evaluate the effects of the different switching compound distributions in a single system.

The "state of the infection" can refer to the number of bacteria in an infection in an infection host, but can also refer to the growth rate of an infecting bacterial population, or to the ability of that infecting population to expand or maintain the infected site in the host animal. Therefore, for example, the state of an infection

has been changed if the numbers (*i.e.*, colony forming units/g of tissue or ml of bodily fluids) of a specific microbe present in the infected animal are reduced, but also if the rate of growth of the population of the microbe has been reduced, even though the absolute numbers have increased. In addition, the state of the infection has been changed if the characteristics of the site of infection have been altered so that the infecting microbe can be more readily eliminated by the host, or so that the microbe is more susceptible to an antimicrobial agent.

In referring to an animal which has been infected with bacteria in the methods of this invention, the "physiological condition" of the animal refers both to the overall health of the animal and to the tissue condition at a localized site affected by the infecting microbe. The general health of the animal may be affected, for instance, by generalized toxins produced by the infecting microbe which are transported throughout the animal. On the other hand, factors produced by the microbial population which contribute to the local death of cells or alteration of their biological functioning also affect the physiological condition of the animal.

In particular embodiments of the above aspects, the recombinant microbe used to infect a mammal is a recombinant bacterium. The bacterial strain used may be any of a large number of pathogenic bacterial species and strains, but in certain preferred embodiments the recombinant microbe is a Staphylococcus species. These Staphylococcus species include, in particular, *Staphylococcus aureus*.

Also in particular embodiments wherein the recombinant bacterium is *Staphylococcus aureus*, the putative pathogenesis gene can be from the *agr* locus, specifically the *hld*/RNAIII coding sequence. As this sequence is known to be a pathogenesis gene, such an embodiment provides a test example for the screening system, as well as providing the capability to determine

the effects of inhibiting the level of activity of the product of this gene under a variety of conditions.

In other preferred embodiments the recombinant bacterium is a *Pseudomonas* species.

In particular embodiments, the recombinant microbe is a virus, where the term "virus" has its usual biological meaning.

In other particular embodiments of the above aspect, the recombinant microbe is a lower eukaryote.

The term "lower eukaryote" refers to eukaryotic organisms which do not form multi-cellular tissues or organs composed of specialized cells. Here, "eukaryote" has its usual biological meaning, indicating an organism whose cells contain a membrane-bounded nuclear compartment distinct from the larger cytoplasm.

In particular embodiments the recombinant microbe is a yeast, or a fungus, or a protozoan. The terms "yeast", "fungus", and "protozoan" have their usual biological meanings.

In certain preferred embodiments, the putative pathogenesis gene or essential gene is derived from a different microbial species than the microbe used for creating the infection. For example, a yeast could contain a gene from a protozoan.

In preferred embodiments in which the recombinant microbe is a bacterium, a putative pathogenesis gene can be from a different bacterial species. These embodiments may be of particular utility for evaluating genes from bacterial species which are difficult to culture or for which there are not acceptable animal infection models.

Similarly, in other preferred embodiments in which the recombinant microbe is a bacterium, a putative pathogenesis gene can be from a yeast, fungus, or protozoan. These embodiments are likewise especially useful when use of the bacterium, fungus, or protozoan would cause technical or analytical difficulties, *e.g.*, if the organism is difficult to culture or if adequate animal

infection models using that specific organism are not available.

In certain preferred embodiments, these methods include the use of recombinant microbes which contain one or more DNA constructs to infect an animal. At least one DNA construct in those microbes contains an artificially controllable promoter inserted in a chromosome. This promoter may by transcriptionally linked with a putative pathogenesis gene or essential gene, such that the expression of that gene, due to transcription from that controllable promoter, can be changed (switched) as described above. The putative pathogenesis or essential gene switched by that promoter can be an endogenous copy of the gene. Having a gene on the chromosome allows more precise control of copy number (and thus dosage effect), which can allow these methods to more closely mimic inhibition of a gene in its normal setting. However, in other embodiments, the chromosomally-inserted promoter controls another element of the switch, such as expression of a repressor molecule. In these embodiments, the test gene may be either chromosomally-inserted, or located in an extra-chromosomal element, such as a plasmid.

The term "artificially controllable promoter" refers to a promoter with properties such that transcription of a transcriptionally linked coding region can be controlled (*i.e.*, switched) experimentally by altering the environment of the microbial cell containing that promoter and coding region. An example of such a change of environment is contacting the microbe with a switching compound. The term "promoter" is used with its usual biological meaning to refer to a DNA sequence which controls the initiation of transcription of a DNA sequence into RNA. (see *e.g.*, Watson et al., MOLECULAR BIOLOGY OF THE CELL, 3rd ed., p.417). Thus, the functions of a promoter can include locating RNA polymerase for starting transcription.

The term "chromosomally-inserted" means that the DNA sequence to which the term refers has been inserted into a chromosome of the microbe by the actions of a person, *i.e.*, it means that the DNA sequence was inserted in the DNA molecule, or one of a set of coordinately replicated DNA molecules, which contain the majority of the genetic information of a cell of the organism. This does not include, *e.g.*, yeast artificial chromosomes (YACs), plasmids, or cosmids.

The term "transcriptionally linked" means that transcription controlled by a specific promoter can proceed across a DNA sequence downstream from that promoter in the same reading frame in which the sequence is transcribed to form a functional RNA in the organism in which the downstream DNA sequence is naturally found.

While the above aspects used mammalian infection models, other animals and organisms can also be used to evaluate test genes from pathogens of those organisms. Thus, in another aspect, this invention provides methods for evaluating a putative pathogenesis gene or essential gene as a target for antimicrobial treatment by infecting a plant with a microbe containing an artificially-created genetic alteration. These methods are therefore similar to the first aspect, described above, except used to evaluate effects of changing the level of activity of a product of a test gene in a plant infection. Particular embodiments of these methods include the use of a switching compound as previously described. As fungi are common pathogens of many food plant crops, in certain embodiments the microbe is a fungus.

In a further aspect, this invention provides recombinant microbial strains in which the microbes express a repressor molecule which enables artificial control of the level of activity of the product of a test gene. In many cases, the repressor is one not normally expressed in the parent strain from which the recombinant microbe was derived, but may also be an endogenous

repressor. The microbial strain also contains an artificially-inserted DNA construct containing an operator site to which the repressor molecule can bind, such that binding of the repressor to the operator site blocks expression of the test gene. In particular embodiments, the construct is chromosomally-inserted, but in other embodiments the construct is carried on an extra-chromosomal element, *e.g.*, a plasmid. Also in particular embodiments, the recombinant microbial strain is a recombinant bacterial strain, such as one derived from a *Staphylococcus* species, like *Staphylococcus aureus*.

In certain preferred embodiments, the recombinant bacterial strain expresses repressor molecules for a *lac* regulatory system and a *bla* regulatory system, such that expression of the *lac* repressor molecule is controlled by the *bla* promoter. Thus, for example, in the absence of a β-lactam which induces the *bla* system, the *bla* repressor blocks expression from the *bla* promoter, which may be linked with the coding sequence for *lac* repressor. The presence of the β-lactam releases the *bla* repressor, allowing expression of the *lac* repressor, which can then block expression of a test gene. (Such as if the promoter for the test gene contains a *lac* operator site.) In a particular preferred embodiment, the strain contains *lacO*, *lacI* transcriptionally linked with P_{blaZ}, and *blaI* and *blaR1* transcriptionally linked with P_{blaR1}. In a particular embodiment, the strain contains a DNA construct which includes the P3 promoter from the *agr* locus of *Staphylococcus aureus*, transcriptionally linked with the *hld*/RNAIII gene from the same locus, and including a *lacO* site. Binding of a lac repressor molecule to the *lacO* site blocks expression of the *hld*/RNAIII gene.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims.

Brief Description of the Figures

Fig. 1 illustrates the use of an *in vivo* switch to mimic the action of an inhibitor of a pathogenesis gene, which in this illustration is *agr*.

Fig. 2 illustrates the working of the *in vivo* switch controlling expression of the *hld*/RNAIII gene in a rodent infection model.

Fig. 3 schematically shows the control of expression of *hld*/RNAIII based on an exemplary selection of DNA constructs and regulatory systems, specifically the *bla* and *lac* regulatory systems. The "inducer" is a switching compound which causes bla repressor to be released from its binding site, allowing expression of lac repressor, which then blocks expression of *hld*/RNAIII.

Fig. 4 schematically depicts DNA constructs suitable for the *in vivo* switch system shown in Figs. 2 and 3. These include the *hld*/RNAIII gene from *Staphylococcus aureus* with its normal promoter, P3, but also including a *lac* operator site. Also included are the coding sequences for the *bla* signal receptor and *bla* repressor, and the *bla* promoter transcriptionally linked with a coding sequence for *lac* repressor.

Fig. 5 illustrates the construction of a DNA construct containing P3, *lacO*, and the *hld*/RNAIII coding sequence.

Fig. 6 illustrates the construction of a DNA construct containing P_{blaZ} transcriptionally linked with *lacI*, and also containing the divergent promoter P_{blaR1}.

Fig. 7 illustrates the construction of a DNA construct in which the coding sequences for *blaR1* and *blaI* are linked with the construct from Fig. 6.

Fig. 8 schematically depicts an embodiment of an *In vivo* switch, in which two different switching compounds can independently cause a change in the level of transcription of a test gene. The switching compounds in this illustration are tetracycline and the β-lactam, CBAP. Either of these compounds can cause the expression of the

lac repressor, thereby causing the expression of the *hld/RNAIII* gene to be switched off.

Detailed Description of the Preferred Embodiments

I. General Description of the Methods

As was described in the Summary above, this invention provides methods for evaluating putative pathogenesis and essential genes as possible targets for antimicrobial treatment by mimicking the effects of agents which directly or indirectly affect the level of activity of the expression product of such a gene. Frequently, these methods are used to model the effect of an inhibitor of the gene product.

In certain preferred embodiments, these methods generally involve contacting a microbe having an artificially-created genetic alteration with a concentration of a molecule which causes a change in the level of production of a second molecule, which alters the level of activity of a product of a specific coding region. Also in certain preferred embodiments, the methods use recombinant microbes which contain one or more DNA constructs, such that the expression of a test gene can be switched by an artificial change in the environment of the microbe. An appropriate change for many embodiments is the addition or removal of a switching compound in the extracellular environment of the microbe. As is further indicated below, genetic switches which respond to a change in the presence of a switching compound can be designed in a variety of ways. One such design of a useful switch is described for use in *Staphylococcus aureus*. In these recombinant microbes, at least one DNA construct has been inserted; that construct contains a coding region of a test gene. (A test gene is a gene which is being evaluated, or is desired to be evaluated, as a potential target for antimicrobial therapy.) The expression of this test gene is either directly or indirectly altered (switched) by the presence

of the switching compound. In certain preferred embodiments, when expression of the test gene is "on", the level of expression of that gene is under the control of the normal cellular mechanisms of the microbe (as in the embodiment exemplified), typically implying that the construct including the test gene also includes the promoter normally associated with that gene. However, such regulation is not essential to the claimed methods.

The control over the expression state may also be achieved using other types or other combinations of constructs. One example is the insertion of specific regulatory regions to artificially control the expression of an endogenous, chromosomally-located gene.

The ability to switch the level of activity of a product of a test gene (as by switching the expression state) makes possible the evaluation of the effect of such a switch *in vivo* (in a microbial infection). In certain of the claimed methods, a recombinant microbe, as generally described above, is used to infect a mammal. At an appropriate time or times, the expression of the test gene is switched by changing the presence of a switching compound and the effect on the infection or the physiological condition of the mammal is evaluated. (Generally the change in the presence of a switching compound is an introduction of that compound, such as by administration to the infected mammal. However, in some cases, the change may be a removal or a decrease in the concentration of the switching compound.) If the switch of the expression of the test gene produces a change in the infection or the condition of the mammal, then the test gene is a possible target for antimicrobial therapy. (Several types of animal infection models are described below.) This is particularly clear if switching off the expression of the test gene results in a significant decrease in the number of microbes present in the infection. In such a case, the switch is mimicking the action of an inhibitor of the test gene or related

products with a possible microcidal effect. However, it should be clear that a demonstration of other effects can also be useful for evaluating potential antimicrobial targets.

Though the discussion above has primarily described the evaluation of genes from pathogens of mammals, it should be understood that it is also desirable to treat microbial infections of some other organisms, specifically including plants. The frequent problems of fungal infections of food crops has led to the widespread use of fungicides for both active treatment of fungal disease, as well prophylacticly. Therefore, the methods of this invention are appropriate and useful for evaluating genes of plant pathogens as targets for antimicrobial treatment in similar manner as described for pathogens of mammals. However, these methods are not limited to mammals and plants, but are applicable to pathogens of many different types of organisms. For pathogens of many non-human organisms, the methods do not require the use of infection models, since the actual infection can be economically and reliably used.

While many of the better-known pathogens appropriate for use in the claimed methods are bacteria whose molecular biology is currently reasonably well-known, it should be recognized that other microbes can also be used. Such use of other microbes can be of several types, including other bacterial species, viruses, yeast, fungi, and protozoans. Organisms which can be properly cultured and whose genes can be suitably manipulated by molecular biology techniques can be utilized in these methods in a manner very similar to the bacteria exemplified.

However, even microbes which are very difficult to culture or to use for recombinant techniques can be used as a source of test genes. Such test genes can then be inserted into a different microbe which is more amenable to manipulation. Thus, for example, genes whose products are suspected of contributing to tissue damage during

infection by a specific difficult to culture microbe can be inserted into a bacterium which can be readily cultured and manipulated. The differential effect of the expression of that heterologous gene can then be evaluated in an infection model of the recombinant bacterium. Therefore, even though a specific microbe may not be suitable for use as the infecting microbe in the methods of this invention, genes associated with the pathogenesis of that specific microbe may still be incorporated in DNA constructs and evaluated as targets.

II. Microbial Strain Selection

Microbial strains are generally chosen for examination in the *in vivo* switch methodology described herein, because of their ability to cause significant disease in mammals (or other host organism of interest). The organism for study can be selected from all microbial pathogens, *e.g.*, bacteria. However, additional factors affect the convenience of utilizing a specific species or strain, and so affect the choice of organisms. (Note that an organism may still be selected despite some difficulties in preparation or use under some circumstances. For example, an important pathogen may be selected for use even if genetic manipulation is difficult.)

To allow for convenient evaluation of the effect of expression and alteration of expression of a test gene, it is preferable that a well-characterized infection model for the specific microbe is available. The infection model should be such that a researcher can distinguish between the development of the pathogenic state and the failure to develop or continue the pathogenic state. The primary criterion for selection of an infection model is the ability of the model to mimic the pathogenic process in the organism(s) which are expected to be treated for infections by that microbe.

Since the microbe is to be used to evaluate the effects of a change in the level of activity of a product of a test gene, such as the effects of a switch of the expression of a test gene, it is in many embodiments highly preferable, but not essential, that the microbe not contain an active copy of the test gene other than the test copy. Thus, the microbial strain used preferably is one in which the normal copy of the test gene has been inactivated or removed. Such inactivation of the normal copy of the gene can occur by any of a variety of different mechanisms. (For examples see Example 2 below.) Of course, in some alternative switch designs, such as one in which an artificially-controllable promoter is inserted in a chromosome, thereby allowing switching of an endogenous gene, the endogenous gene should not be inactivated.

In addition, the microbial strain should preferably be amenable to manipulation using molecular biological methods, and putative pathogenesis or essential genes (test genes) must be available for testing. In most cases, this implies that the test gene will need to be isolated for further manipulation. Such manipulation generally comprise the construction of one or more DNA constructs which include a significant portion of the test gene, but may comprise other processes such as single base changes of DNA sequences. Likewise, the microbial strain which is used in the animal, or other, infection model is preferably one in which a recombinant DNA construct can be introduced into the microbial cell.

Once an appropriate strain is selected, it is possible to create a set of receptor strains derived from the initial strain which provide a "cellular cassette system" for test genes. Such receptor strains are particularly useful since they provide a convenient means to provide strains to test a variety of different test genes, or to test genes which can be switched using a variety of different environmental changes, since the new

sequences can be inserted more readily than if new constructs, insertion sites, and/or vectors were created for each test. Thus, each derivative strain can contain a specific promoter with a downstream insertion site for test genes to be controlled by that promoter. As another alternative, a site can be constructed for insertion for a complete promoter/test gene construct. Such a set of receptor strains facilitates the evaluation of potential targets.

The use of the selection factors appropriate for choosing a microbe to use as a recombinant microbe in the *in vivo* switch methodology of this invention is described for a bacterium, *Staphylococcus aureus*, in Example 1 below. It should be noted, however, that the species and strain described, while appropriate for such use, are in no manner limiting to the claims. Other species can appropriately be selected, and other equally appropriate strains of *Staphylococcus aureus* can be selected or constructed using standard techniques of molecular biology.

Example 1: Selection of Recombinant Microbe - *Staphylococcus aureus*

One bacterial species selected for examination is *Staphylococcus aureus*. This bacterium is chosen because of its status as a common, but often difficult to treat, microbial pathogen. *S. aureus* has the acquired ability to be resistant to a variety of antibiotics, and has also adapted well to being a human pathogen, including the ability to persist in a carrier state (as in the nasal passages of health care workers). In some cases today, only glycopeptides (vancomycin and, in Europe, teicoplanin) are recommended as reasonable therapies.

This organism is genetically tractable, capable of manipulation through the use of genetic and recombinant technologies. The molecular biology and some elements of the pathogenesis of this gram-positive species are relatively well-characterized. The genetic tractability

and the partial characterization of the pathogenesis of *S. aureus* means that both known pathogenesis genes as well as test genes can be isolated and manipulated by well known molecular biology techniques. In addition, DNA constructs can be re-introduced into *Staphylococcus aureus* by currently available methods, including both chemical treatment of cells to induce competence, as well as introduction of DNA constructs by electroporation. *S. aureus* is a classic representative of the eubacteria, and understanding more about its ability to cause disease will clearly provide a significant foundation for understanding other bacteria.

Thus, the criteria for choosing *S. aureus* as an example of a appropriate recombinant microbe include: (1) ability to cause significant and common human disease; (2) genetic accessibility; and (3) representative character (for extension to other microbes).

However, within a microbial species certain strain characteristics can be significant. The *in vivo* switch provides the capability to artificially control the expression of a test gene with a small molecule switching compound. The recombinant construction that effects this control is preferably introduced into a mutant strain that lacks expression of active product from that gene (test gene or natural homolog) apart from the control of the *in vivo* switch. This allows the sole expression of the test gene from the recombinant construction.

Staphylococcus aureus, as found in wild-type clinical isolates, often carries extrachromosomal genetic elements (both plasmids and transposons). The use of such strains would complicate the analysis of the results of testing, as insertions into the chromosome, or the existence of extrachromosomal elements, is variable within clinical isolates. Hence, a strain devoid of extrachromosomal elements is desirable for prototypic study.

Strain 8325-4 is such a strain. Genetic mapping has been done on the strain; it is thought to be devoid of

transposons, plasmids and phage. However, it is believed to be otherwise representative of *S. aureus*. An *in vivo* switch controlling expression of the *agr-RNAIII* gene uses the *S. aureus* strain WA400 (Cm^R; Strain 8325-4 with Δhld/RNAIII 252-1472::*cat-86* (S. Arvidson et al., Ch. 30 p.419 ff in MOLECULAR BIOLOGY OF THE STAPHYLOCOCCI, R.P. Novick, ed., VCH, New York, New York, 1990). The WA400 Agr phenotype is readily complemented by an extra-chromosomal copy of the *agr* region spanning BgIII 1-PstI 2149. The intended recombinant construction is also expected to fully complement the *Agr* defect of this mutant strain. Other such strains are available.

III. Selection of Inserted Genes

Two general types of putative pathogenesis genes are isolated into the switch construct. First are genes suspected of being involved in microbial pathogenesis. These genes are identified using a variety of techniques, including the following approaches. (1) Some pathogenesis genes are identified by reviewing the literature. (2) A second group is identified by using techniques which select genes which are expressed or essential specifically during infection. Such techniques include but are not limited to the techniques of differential display (Liang & Pardee, 1992, *Science* 257:967-971), differential hybridization (T. Sargent, 1987, *Meth. Enz.* 152:423-432), or IVET (*In vivo* Expression Technology) (J. Mekalanos, 1993, *J. Bacteriol.* 174:1-7). In each of these techniques, cells are grown either in animals or under conditions which mimic such *in vivo* growth. Conditions which are commonly accepted as mimics of *in vivo* growth include, but are not limited to, iron deprivation, late exponential (vs. exponential) growth; conditions of oxygen limitation and other forms of nutrient limitation. (3) A third group of pathogenesis genes is identified by observing differential virulence in strains in which specific genes have been interfered with (made

nonfunctional or differentially functional). Using animal models such as described below in In vivo evaluation of microbial virulence and pathogenicity, lack of virulence in such models serves to identify potential pathogenesis genes. (4) It is becoming more commonly accepted that in bacteria, individual pathogenesis genes may reside near other genes which share similar functions. Such clusters of genes involved in pathogenesis have come to be known as "pathogenesis islands". Pathogenesis islands clearly exist in enteric bacilli such as *Salmonella*. (E.A. Groisman, 1993, *EMBO J.* 12:3779-37787; Galan & Curtiss, 1989, *PNAS* 86:6383-6387.) Using the assumption that an identified pathogenesis gene may have neighbors which are also important in pathogenesis, genes neighboring identified pathogenesis genes are also chosen for inclusion in switch experiments. Thus, even genes showing no homology or very little homology to studied genes are chosen for study using the switch based on their locations within the genome (having pathogenesis gene neighbors). (5) Genes which are essential for microbial growth or survival *in vivo* are also utilized. The expression of these genes is required *in vivo*, but not *in vitro*.

A second major collection of genes which is studied using the in vivo switch is so-called "essential genes". Essential genes are described as genes which, if not present in a functional state, result in the death of the microbe. Such genes can be identified by a variety of techniques, (S.J. Austin et al., 1971, *Nature* 232: 133-136; P. Schedl & P. Primakoff, 1973, *Proc. Natl. Acad. Sci.* 70: 2091-2095; C.M. Joyce & N.D.F. Grindley, 1984, *J. Bacteriol.* 158: 636-643) but one straightforward and proven method is through the use of temperature sensitive mutants. Mutants, created for example by exposure to mutagenic chemicals, are selected for their ability to grow at a permissive temperature, but not a non-permissive temperature. It has been demonstrated in some cases, and is widely accepted, that such mutants have changes in

their DNA which makes the mutated gene products differentially active at the different temperatures. For example, a mutant may have a functional enzyme at thirty degrees centigrade, but a dysfunctional enzyme at 37° centigrade. The dysfunction may be due to changes in the enzyme brought about by the mutation in the DNA. In such an event, if the enzyme's function is required (essential) for the ability to grow, we have identified an essential gene (for those growth conditions). Identification of these essential genes *in vitro* does not guarantee that the identified genes are essential for viability *in vivo*, or that disruption of the expression of an essential gene has the same quantitative or qualitative effect *in vivo*. Hence, essential genes selected *in vitro* are included for examination in the switch model. This examination allows confirmation that the "essential gene" is, in fact, essential for the cell's viability *in vivo*. Controlling the expression of such genes while the bacteria is within an animal provides definitive evidence for the essential nature of such genes during infection, and as such allows for an appreciation of the importance of the (*in vitro* selected) essential gene. This allows evaluation of the relevance of the gene as an antimicrobial target, and therefore provides a basis for selection of appropriate genes to use for the development of an antimicrobial agent.

Genes identified as essential genes need not be previously known, nor do the products of those genes, or the functions of those products need to be known. The genes may be known "only" as open reading frames, the expression of which correlates with *in vitro* microbial viability under the tested growth conditions.

Another approach to the identification of either pathogenesis or essential gene targets is a "shotgun approach". While different methods could be utilized, one such method is the integration of an artificially controllable promoter into the microbial chromosome.

(These techniques are most developed for bacteria, both Gram-positive and Gram-negative.) If inserted in the proper reading frame, the artificially controllable promoter can switch the expression of an endogenous gene, replacing the normal cellular control of the gene. This method is particularly appropriate for identifying genes whose expression is deleterious to the microbe *in vivo*, *i.e.* in an infection. Thus, a controllable promoter on a DNA integration vehicle (*e.g.*, insertion sequence or transposon) can be inserted in the chromosomal DNA at a large number of sites.

This allows the isolation of a large number of derivative strains; in each the expression of a gene can be artificially controlled. Artificial control over the expression of a random set of genes then allows determination of the effects of those gene products on the progression of an infection. (This shotgun approach does not rely on any prior knowledge of the probable function of a gene.)

In summary, both pathogenesis genes and essential genes are placed in the artificially-controllable system. ORF's identified as neighbors of identified pathogenesis genes or identified as essential genes are included. Pathogenesis and essential genes which are identified from the literature are also included as are genes which enhance virulence (as identified in animal modeling). Further, differentially expressed genes (genes which are expressed specifically during infection) are also candidates for examination in the switch model. Alternatively, in the shotgun approach using chromosomally-inserted promoters, a random set of genes can be evaluated.

In addition to the evaluation of a single test gene, this invention also allows (and the claims include) evaluation of a set of genes together. For example, a linked set of genes, such as an operon with multiple open reading frames can be evaluated by placing the level of

activity of all the coding sequences under the same control, such as transcriptional control. As another possibility, a set of unlinked genes can be subjected to the same control. This can be accomplished, for example, by using the same type of operator site with each coding sequence to bind a single type of repressor, allowing all the test genes to be turned off at essentially the same time.

For the microbe described in Example 1 above, *Staphylococcus aureus*, a number of pathogenesis genes are currently known. These known genes provide examples of genes which are appropriate to evaluate as targets using the *in vivo* switch methodology, and some are further described in Example 2 below.

Example 2: *Staphylococcus aureus* Pathogenesis Genes

For *Staphylococcus aureus*, a number of pathogenesis genes are known. These known genes include *agrA*, *agrB*, *agr-RNAIII*, *xpr*, and *sar*. Of these genes, several are part of the *agr* locus, which is a complex, polycistronic locus which controls the production of most cell toxins and many cell wall-associated proteins. The biosynthesis of these gene products involves both positive and negative regulation. The *agr* locus contains two divergent promoters, P2 and P3, which are strongly induced during post-exponential growth. Promoter P3 directs the synthesis of a 514 nt transcript RNAIII which contains the *hld* (delta-lysin) open reading frame. Recent evidence indicates that the RNAIII molecule, in addition to encoding delta-lysin, also encodes the *agr*-specific regulator, since the loss of RNAIII production results in altered regulation of exoprotein virulence factors. The gene expression regulated by RNAIII involves virulence factors which are up-regulated, as well as virulence factors which are down-regulated.

IV. Inactivation of Endogenous Copy of Test Gene

In many embodiments in which a test gene coding region has been inserted in a microbe, expression of an endogenous gene corresponding to the test gene would interfere with the evaluation of the test gene. Therefore, in those embodiments, it is preferable that the endogenous gene is inactivated. Such inactivation can be achieved in various ways. Examples of such inactivation mechanisms are, *e.g.*, insertional disruption of function (such as with a transposon or other insertion sequence); by an insertion/deletion (in which DNA has been replaced with inserted DNA); or by a spontaneous or induced mutation in the test gene (such as by chemically or uv-induced mutagenesis).

A specific example of gene inactivation using recombinant DNA methods is allelic replacement. In allelic replacement, the native chromosomal allele of a given gene is replaced, using host cell general recombination factors, with a nonfunctional gene copy. Nonfunctional alleles of a given gene are created *in vitro* by insertion/ disruption or insertion/ deletion within the open reading frame of a test gene. The insertion sequence contains both the promoter and coding region for a drug resistance marker (eg. for tetracycline, *tetM*; for chloramphenicol, *cat-86*; for erythromycin, *ermC*), thus enabling *in vivo* selection for gene replacement by acquisition of drug resistance. Examples of the application of this method in *S. aureus* are:

a) toxic shock syndrome toxin gene (tst): Sloane et al., 1991, *FEMS Microbiology Letters* 78: 239-244.

b) accessory gene regulator locus (agr): Novick et al., 1993, *EMBO J.* 12: 3967-3975.

c) delta hemolysin gene (hld/RNAIII): Janzon and Arvidson, 1990, *EMBO J.* 9: 1391-1399.

V. Regulatory Mechanisms and Selection of Regulatory Sequences

Many genes in microbes are regulated by transcriptional control. They are switched on and off in a conservative fashion, being transcribed when the products are needed, and not transcribed or transcribed at a lower level when the products are not needed. Such regulation is advantageous for a number of reasons, including reducing unproductive energy usage by limiting the synthesis of unneeded molecules, and reducing the competition between conflicting cellular processes.

While transcriptional control is an important mechanism, regulation of the level of activity of a gene product in microbes can occur through a variety of mechanisms. These include regulation of the production of mRNA transcripts, of the intracellular longevity of the mRNA, of the ability to translate the mRNA into protein, of the processing of the protein into a functional form and/or of the intracellular stability of the protein. Each species uses some or all of these mechanism for regulating the level of activity of gene products. Likewise, each of these mechanisms can be used as the basis of an *in vivo* switch, as disclosed herein, to control the level of activity of the product of a test gene. Certain preferred embodiments of the methods of this invention achieves gene regulation by control over synthesis of the mRNA, *i.e.*, over transcription.

Control of the synthesis of RNAs is accomplished by the synthesis of regulatory proteins that serve to make the DNA template more or less accessible to the RNA polymerase enzyme. The study of transcriptional control in microbes, especially in bacteria, has a long history; general features of the molecular mechanisms underlying transcriptional control in bacteria have been under continuous investigation since the early 1960s. Summaries of this work can be found in any undergraduate textbook on molecular biology (*see e.g.*, Watson, et al., Molecular

Biology of the Gene). In particular, note that the definitions of the terms "operator", "promoter", "repressor protein", and "inducer protein" can be found in those textbooks. (The description immediately below is not intended to be comprehensive, and generally discusses bacterial gene regulation. It should be recognized that different microbial groups may have features of gene regulation unique to each group, and indeed, each species with a group may have unique elements. Nonetheless, major elements of the process are common to all the microbes.)

Regulatory proteins modify the ability of RNA polymerase to synthesize the mRNA of one or more physiologically related genes. They accomplish this task by physically binding to the DNA in the vicinity of the promoter site, at which RNA polymerase binds and initiates synthesis of the mRNA. When a regulatory protein binds and prevents or reduces the ability of RNA polymerase to synthesize a mRNA, it is called a repressor protein. The binding of the repressor protein to the DNA occurs at a site at which the repressor protein has a particularly high affinity. This high affinity is achieved through the interaction of the protein with the specific sequence of nucleic acid base pairs in the DNA duplex, typically through a combination of H-bonding, van der Waals interactions, hydrophobic interactions, and ionic interactions. This binding site, called the operator site (which is generally 8-30 bp), is specific for each different repressor protein. Likewise, there are regulatory proteins, inducers, that bind to a specific DNA sequence of base pairs that induce RNA polymerase to synthesize a mRNA transcript.

The DNA-binding activity of repressor proteins and inducer proteins is regulated in response to changes in the bacterial cell physiology. In many cases, small molecules (such as cAMP, sugars and other metabolites) bind to the regulatory protein causing an altered affinity of the regulatory protein for the DNA.

The sensing mechanisms which stimulate the induction and repression of genes include microbial capability to accept and transmit stimuli from the outside world. Environmental sensors can create responses from the microbe to diverse stimuli, including presence of specific nutrients, the lack of nutrients, metals and cofactors, the presence of antibiotics and changes in the external environment. In this last case, examples include changes in temperature (*e.g.*, heat shock genes) and gases (e.g. oxygen). Two well-studied prototypic environmental sensors are the lactose operon and the tetracycline resistance operon.

Selection of appropriate regulatory sequences (including those providing environmental sensor function) to use in the methods of this invention depends on a number of considerations. An approach to such regulatory sequence selection with some of its associated considerations is described in (a) through (g) below.

(a) A promoter is required that will regulate the expression of the test gene. In the exemplified strategy, an "off" switch has been designed. In this design, a small molecule is introduced into the animal, and the test gene is no longer expressed.

(b) A promoter that is regulated to turn-off when a small molecule is introduced is the simplest mechanism to control the expression of the test gene. However, the best promoter provides expression at appropriate levels *in vivo*, and this level of expression may differ for each test gene. Therefore, there is good reason to provide artificial control of the natural promoter of the test gene. However, use of the natural promoter is not essential. In many cases, a heterologous promoter may be linked with the test gene, and in some cases the use of an heterologous promoter will be preferable or even necessary. Such situations may arise, for example, when evaluating a test gene from an organism other than the infecting organism, particularly in those cases where

normal control of transcription relies on a number of additional components.

(c) Regulation of the natural promoter of the test gene can be accomplished by introducing a negative regulatory element in *cis* to the test gene and promoter, in such a position and orientation that a *trans*-acting negative regulatory protein can prevent expression. An example of such a *cis/trans* regulatory pair is the *lacO* operator site and the *lac* repressor protein. (Amann, et al., 1983, *Gene*, 25: 167-178; Makoff and Oxer, 1991, *Nucl. Acid Res.* 19: 2417-2421). Other examples of negative regulatory pairs are the *tet* operator/*tet* repressor; the lambda cI repressor/lambda O_L operator; (Bernard, et al., 1979, *Gene* 5: 59-76; Mott, et al., 1985, *Proc Natl. Acad. Sci (USA)* 82: 88-92); *trp* repressor/*trp* operator (Latta, et al., 1990, *DNA & Cell Biol.* 9: 129-137); *argR* repressor/*arg* operator (Lim, et al., 1987, *Proc. Natl. Acad. Sci. USA* 84: 6697-6701); *lexA* repressor/*boxA* operator site (Kenyon, et al., 1982, *J. Mol. Biol.* 160: 445-457); the *bla* repressor/*bla* operator (Novick, et al., 1991, *EMBO J.*). There are many others. The operator/repressor pairs need not originate in the pathogen under investigation, since many studies have shown that effective regulation can occur independent of the species of origin of the regulators. In a specific embodiment described herein, the *E. coli lac* operator/*lac* repressor regulatory system can be used to control the expression of the *agr*-related *hld/RNAIII* gene of *S. aureus*.

(d) In order to achieve regulation of the test gene that has been placed under artificial control of a negative regulatory system, artificial control of the *trans*-acting repressor is required. A preferred strategy is to control expression of the repressor protein. The repressor protein should be synthesized in response to addition of a small molecule to the animal. This can be achieved by a promoter that is turned on by the addition

of a small molecule switching compound. Such promoters are either positive activators, in which the small molecule makes a *trans*-acting factor allow transcription from a promoter, or by a negative mechanism, in which the small molecule makes a *trans*-acting repressor protein no longer capable of binding to its operator site. There are numerous examples of positive and negative regulators. Several negative regulator systems were listed in (c); positive acting systems include the activation of promoters by the CAP protein in the presence of cAMP and the activation of the *ara* promoter by *araC* protein in the presence of arabinose. The coupling of these artificial regulatory constructions allows a small molecule to control the expression of a regulatory protein that controls the expression of the test gene. In an example described herein, a β-lactamase system controls expression of a *lac* repressor protein, and the test gene is linked with a lac operator site in *cis*. Therefore, exposing a bacterial cell containing this system to the non-antibiotic β-lactam CBAP causes the β-lactamase repressor to be released, resulting in the expression of the lac repressor, blocking transcription of the test gene.

(e) A different strategy is to regulate expression by an inversion mechanism. There are several examples of site-specific inversions that occur in which the inverted segment includes a promoter. The expression of a test gene is on in one orientation of the promoter, and off in the other orientation of the promoter. The inversion is controlled by a site-specific recombinase enzyme; control over expression of the test gene is accomplished by controlling expression of the recombinase enzyme by a small molecule switching compound, such as described in (c) and (d). Addition of the small molecule results in expression of the recombinase enzyme, which shuts off expression of the test gene. Examples of this strategy are *tnpR* resolvase-mediated inversion of *γδ-res* sequences, *E. coli P1cre-lox* mediated inversion, and bacteriophage λ

Int-mediated inversion of *att* sequences. (Podhajaska et al., 1985, *Gene* 40: 163-168.)

Another strategy involves antisense RNA. Numerous genes in prokaryotes are regulated by antisense RNA (Simons and Kleckner, 1988, *Ann. Rev. Genet.* 22:567-600; Wagner and Simons, 1994, *Ann. Rev. Micorbiol.* 48:713-742). The expression of a test gene is down regulated by induction of an antisense RNA complementary to the test gene transcript. Expression of the antisense RNA can be accomplished by a small molecule inducer system such as described in (c) and (g). Alternatively, in an approach independent of production of a regulatory molecule by the microbial cell, antisense oligonucleotides can be administered which are chemically modified to provide increased biological stability, but which can bind to a test gene transcript to down regulate expression. (Uhlmann & Peyman, 1990, *Chem. Rev.* 90:544-584.)

Yet another strategy is to utilize ribozymes to control expression by cleaving the mRNA transcribed from the test gene. A sequence(s) coding for a ribozyme can be introduced into a microbe such that expression of the ribozyme(s) is induced by addition of a small molecule (such as the tetracycline of β-lactamase-inducible promoters). In this system, expression of the ribozymes would be off until the small molecule (switching compound) is added. The presence of the ribozymes then reduces expression of the test gene. Alternatively, ribozymes may be utilized by administering modified ribozymes which are more stable than RNA ribozymes in a form which can be taken up by the microbe. (Eckstein et al., 1992, PCT Application PCT/EP91/01811, Int. Publ. No. WO92/07065.) In this case, the ribozymes can act directly on the test gene message.

(f) The promoter must respond in its expression to a small molecule that retains bioavailability in the relevant tissue of fluid in the animal model. The best promoters have normal expression of the test gene in the

absence of the small molecule, and a complete cessation of expression of the test gene in the presence of the small molecule.

(g) The best small molecules to regulate the promoter expression are those that have well-defined pharmacodynamics. Such well-defined pharmacodynamics allow assessment of the effects of the small molecule alone (there should be very little effect, such as growth inhibition at the doses used). In addition, the pharmacodynamic information assures that the necessary concentrations of the small molecule are achieved in the tissue under investigation in the animal model, so that the test gene is appropriately regulated. As discussed above, different compounds will distribute differently, and have different tropism and clearance characteristics in various tissues, so that the effective concentration of the compound at a specific infection site will vary. Due to these variable properties, the temporal behavior of various switching compounds will differ, with accompanying differences in the control of the level of activity of a gene. Some such compounds are known among the inactive (or low activity) analogs of known antibiotics. For these compounds, the pharmacodynamics of the class of compounds has been previously investigated, but the inactive analog does not have significant antimicrobial activity to complicate the evaluation of targets. In addition, the use of different switching compounds which have varying access to different tissues can provide additional information on the suitability of a target in relation to dissemination of the infection, and of microbial load in the different tissues. In certain cases, a range of switching compounds can be used with one coding region (and with appropriate regulatory components) in order to evaluate the effects of the different switching compound distributions in a single system. In general, however, a switching compound should be chosen which has pharmacodynamic properties appropriate for use with a

specific test gene and infection model. For example, the switching compound must distribute to the infection site with sufficient concentration and half-life to effectively switch the level of activity of the gene.

VI. Genetic Methods and DNA Constructs

For use in evaluating a putative pathogenesis or essential gene, one or more DNA constructs are made and inserted in a test microbe (*e.g.*, *Staphylococcus aureus*). The methods for performing these steps are well-known in the art, and can be selected or modified from those described in such standard references as Sambrook et al., MOLECULAR CLONING, 2nd ed. (1989).

Typically, but not necessarily, two constructs are made. In one class of embodiment of this invention, the first construct contains a regulatory region which is controlled by the presence or absence of a small molecule, transcriptionally linked with a coding region for a signal molecule which can control the expression of the second construct. The second construct includes a regulatory region which is controlled by the normal mechanisms of the cell, an operator region which is controlled by the product of the coding region of the first construct, and the coding region of a test gene.

While the paragraph above generally describes the constructs for one class of embodiment, it does not describe all the useful designs. It is only necessary that the level of activity of the product of a test gene can be switched (as by switching expression) in response to the addition or removal of a switching compound or other appropriate environmental change. However, as previously indicated, in many situations it is advantageous for the level of expression of the test gene to be under the normal control of the microbial cell when the expression state is on.

The example below provides a description of one preferred embodiment of DNA construct design for

Staphylococcus aureus, but should not be regarded as limiting even for this species. With these constructs the small molecule CBAP (a penicillin derivative with well-known pharmacodynamics) causes the *bla* repressor protein to lose its affinity for the *bla* operator site. This allows expression of the *lac* repressor protein, which blocks the expression of the *agr-RNAIII* molecule. The RNAIII molecule is important for the pathogenesis of *S. aureus*. (M. Richmond, 1967, *J. Mol. Biol.*, 26: 357.)

The example below describes DNA constructs which are inserted into the bacterial chromosome, however, chromosomal insertion is not necessary. Therefore, in another preferred embodiment, the same switch components are used but only one construct is inserted in the chromosome. The P_{bls2}-*lacI* fusion is inserted in the chromosome, and the P3-*lacO-hld*/RNAIII and *blaR1-blaI* sequences are placed in a plasmid which remains independent of the chromosome. An advantage of this embodiment is ease of construction, since plasmid insertions are generally simpler to accomplish than chromosomal insertions. Embodiments utilizing plasmids in this manner require that the plasmid by stable within the bacterial cells; if a specific plasmid is lost at an excessive frequency then either a different plasmid or chromosomal insertion is preferable.

EXAMPLE 3: DNA Constructs for *Staphylococcus aureus* - *agr locus*

1. Construction of hybrid *S. aureus* P3-*lacO*-RNAIII promoter (see Fig. 5) :

(a) Subclone *hld*/ RNAIII 2149 bp fragment (PstI - BglII, from pEX07; Janzon and Arvidson) into PstI site of pALTER-1 (Promega) for oligonucleotide mediated site-directed mutagenesis.

(b) Anneal mutagenic oligonucleotide (PIVS5) to convert C1557 to G, creating unique BglII site at position +13 relative to *hld* transcription start point (tsp; = 1570).

(c) Insert synthetic 21 bp core lacO oligonucleotide (PIVS6 + 7) at BglII.

(d) Select 3 lacO configurations: 1 oligo forward, 1 oligo reverse, and 3 tandem forward oligo insertions.

(e) Transfer to *S. aureus - E. coli* shuttle vector pMP157, to create plasmids pMPswitch7/8/9, respectively, and *S. aureus* integration vector pXX.

2. Construction of inducible *E. coli lacI* gene in *S. aureus*

(a) Fuse *S. aureus blaZ* promoter (P_{blaZ}) to *E. coli lacI* coding sequence by polymerase chain reaction (PCR); transfer to S. aureus - E. coli shuttle vector pMP16, to create plasmid pMPswitch3, and to *S. aureus* integration vector pXXX (see Fig. 6).

(b) Subclone genes encoding beta-lactamase repressor (*blaI*) and beta-lactamase signal receptor (*blaR1*) into pMPswitch3, to create plasmid pMPswitch4, and to *S. aureus* integration vector pXXX (see Fig. 7).

3. Transfer plasmids containing P3-*lacO*-RNAIII (pMPswitch7/8/9), and *blaI-blaR1*-P_{blaZ}-*lacI* (pMPswitch4) into *S. aureus* strain WA400 (8325-4 Δ*hld*/RNAIII 252-1472::*cat-86*; Janzon and Arvidson) by electroporation.

VII. *In Vitro* Evaluation of DNA Construct Function

Once the DNA constructs have been made for a selected organism and test gene, it is useful to test the *in vitro* functioning of the regulation and expression of those constructs. Such verification of function avoids wasteful use of animals in infection studies, and as well can provide greater confidence in the results of evaluation of test genes using the constructs.

The exact function tests appropriate for a specific DNA construct or set of constructs will, of course, depend on the specific switch design, as well as on the specific regulatory sequences, control molecules, and coding sequences involved. However, the appropriate tests are directly implied by the switch design, so the nature of the appropriate testing is neither ambiguous nor difficult. As an example, the testing of the constructs described in Example 3 above, for use with a *Staphylococcus aureus* strain, is described in Example 4 below.

Example 4: *In vitro* Testing of DNA Constructs

In vitro evaluation

(1) A biochemical assay for *lacO* function is performed. A gel band shift assay, employing labeled or tagged *lacO* oligonucleotide and extracts derived from induced and non-induced recombinant *S. aureus* containing the P_{blaZ}-*lacI* construct is performed. The sensitivity of this method is sufficient to detect induced levels of *lacI*, but low levels of the *lac* repressor synthesis are generally undetectable by this method.

(2) Appropriate Agr-regulation of the P3-*lacO*-RNAIII promoter.

The insertion of *lacO* into the *agr* P3 promoter must not alter the appropriate regulation of the *hld*/RNAIII transcript by the agr regulation system. The appropriate induction of RNAIII during postexponential growth is tested by isolation of RNA from recombinant strain WA400:P3-*lacO*-RNAIII and subsequent RNA blot hybridization analysis to identify the RNAIII transcript.

(3) Efficacy of "Off-Switch" *in vitro*:

The effect of *lac* repressor induction on RNAIII accumulation in recombinant strain WA400:P_{blaZ}-*lacI*; *blaI*; *blaRI*; P3-*lacO*-RNAIII is assessed in cell culture by RNA blot hybridization as well as by various genetic and biochemical tests (hemolysin and lipase production; P3-*blaZ induction, etc.*).

VIII. In vivo evaluation of microbial virulence and pathogenicity

After the genetic constructs have been placed into their host organisms, they are evaluated in an infection model system, *e.g.*, in an animal, cell-based, or plant system. (References herein to the use of an animals or mammals should be understood to refer to particular embodiments of this invention. As mentioned above, other infection systems may be used for certain other embodiments, such as to evaluate possible antimicrobial targets of pathogens of organisms other than animals (*e.g.*, plants), and to embodiments employing cell-based systems as surrogates for whole organism models.) The criteria for evaluation include the ability of the microbe to replicate (either with test gene expression "on" or test gene expression "off"), the ability to produce specific exoproducts involved in virulence of the organism, and the ability to cause symptoms of disease in the animals.

The infection models, *e.g.*, animal infection models, are selected primarily on the basis of the ability of the model to mimic the natural pathogenic state of pathogen in an organism to be treated and to distinguish the effects produced by a change in the level of activity of a gene product (*e.g.*, to a switch in the expression state of the gene). Secondarily, the models are selected for efficiency, reproducibility, and cost containment. For mammal models, rodents, especially mice, rats, and rabbits, are generally the preferred species. Experimentalists have the greatest experience with these species. Manipulations are more convenient and amount of materials which are required are relatively small due to the size of the rodents.

Each pathogenic microbe (*e.g.*, bacterium) used in these methods will likely need to be examined using a variety of infection models in order to adequately

understand the importance of the function of a particular test gene.

A number of animal models suitable for use with bacteria are described below. However, these models are only examples which are suitable for a variety of bacterial species; even for those bacterial species other models may be found to be superior, at least for some inserted genes and possibly for all. In addition, modifications of these models, or perhaps completely different animal models are appropriate with certain bacteria, as well as with species of other types of organisms, *e.g.*, yeast, fungi, and protozoa.

Six animal models are currently used with bacteria to appreciate the effects of switching on and off specific genes, and are briefly described below.

Example 5: Mouse Soft Tissue Model

The mouse soft tissue infection model is a sensitive and effective method for measurement of bacterial proliferation. In these models (Vogelman et al., 1988, *J. Infect. Dis.* 157: 287-298) anesthetized mice are infected with the bacteria in the muscle of the hind thigh. The mice can be either chemically immune compromised (*e.g.*, cytoxan treated at 125 mg/kg on days -4, -2, and 0) or immunocompetent. The dose of microbe necessary to cause an infection is variable and depends on the individual microbe, but commonly is on the order of 10^5 - 10^6 colony forming units per injection for bacteria. A variety of mouse strains are useful in this model although Swiss Webster and DBA2 lines are most commonly used. Once infected the animals are conscious and show no overt ill effects of the infections for approximately 12 hours. After that time virulent strains cause swelling of the thigh muscle, and the animals can become bacteremic within approximately 24 hours. This model most effectively measures proliferation of the microbe, and this proliferation is measured by sacrifice of the infected animal and counting colonies from homogenized thighs.

Example 6: Diffusion Chamber Model

A second model useful for assessing the virulence of microbes is the diffusion chamber model (Malouin et al., 1990, *Infect. Immun.* 58: 1247-1253; Doy et al., 1980, *J. Infect. Dis.* 2: 39-51; Kelly et al., 1989, *Infect. Immun.* 57: 344-350. In this model rodents have a diffusion chamber surgically placed in the peritoneal cavity. The chamber consists of a polypropylene cylinder with semipermeable membranes covering the chamber ends. Diffusion of peritoneal fluid into and out of the chamber provides nutrients for the microbes. The progression of the "infection" can be followed by examining growth, the exoproduct production or RNA messages. The time experiments are done by sampling multiple chambers.

Example 7: Endocarditis Model

For bacteria, an important animal model effective in assessing pathogenicity and virulence is the endocarditis model (J. Santoro and M.E. Levinson, 1978, *Infect. Immun.* 19: 915-918). A rat endocarditis model can be used to assess colonization, virulence and proliferation. The ability to switch specific genes on and off has clear utility using this model.

Example 8: Osteomyelitis Model

A fourth model useful in the valuation of pathogenesis is the osteomyelitis model (Spagnolo et al., 1993, *Infect. Immun.* 61: 5225-5230). Rabbits are used for these experiments. Anesthetized animals have a small segment of the tibia removed and microorganisms are microinjected into the wound. The excised bone segment is replaced and the progression of the disease is monitored. Clinical signs, particularly inflammation and swelling are monitored. Termination of the experiment allows histolic and pathologic examination of the infection site to complement the assessment procedure.

Example 9: Murine Septic Arthritis Model

A fifth model relevant to the study of microbial pathogenesis is a murine septic arthritis model (Abdelnour

et al., 1993, *Infect. Immun.* 61: 3879-3885). In this model mice are infected intravenously and pathogenic organisms are found to cause inflammation in distal limb joints. Monitoring of the inflammation and comparison of inflammation vs. inocula allows assessment of the virulence of related strains.

Example 10: Bacterial Peritonitis Model

Finally, bacterial peritonitis offers rapid and predictive data on the virulence of strains (M.G. Bergeron, 1978, *Scand. J. Infect. Dis.* Suppl. 14: 189-206; S.D. Davis, 1975, *Antimicrob. Agents Chemother.* 8: 50-53). Peritonitis in rodents, preferably mice, can provide essential data on the importance of targets. The end point may be lethality or clinical signs can be monitored. Variation in infection dose in comparison to outcome allows evaluation of the virulence of individual strains.

A variety of other *in vivo* models are available and may be used when appropriate for specific pathogens or specific genes. For example, target organ recovery assays (Gordee et al., 1984, *J. Antibiotics* 37:1054-1065; Bannatyne et al., 1992, *Infect.* 20:168-170) may be useful for fungi and for bacterial pathogens which are not acutely virulent to animals. For additional information the book by Zak and Sande (EXPERIMENTAL MODELS IN ANTIMICROBIAL CHEMOTHERAPY, O. Zak and M.A. Sande (eds.), Academic Press, London (1986) is considered a standard.

It is also relevant to note that the species of animal used for an infection model, and the specific genetic make-up of that animal, may contribute to the effective evaluation of the effects of altering the level of activity of the product of a test gene. For example, immuno-incompetent animals may, in some instances, be preferable to immuno-competent animals. For example, the action of a competent immune system may, to some degree, mask the effects of altering the level of activity of the test gene product as compared to a similar infection in an immuno-incompetent animal. In addition, many

opportunistic infections, in fact, occur in immuno-compromised patients, so modeling an infection in a similar immunological environment is appropriate.

In addition to these *in vivo* test systems, a variety of *ex vivo* models for assessing bacterial virulence may be employed (Falkow et al., 1992, *Ann. Rev. Cell Biol.* 8:333-363). These include, but are not limited to, assays which measure bacterial attachment to, and invasion of, tissue culture cell monolayers. With specific regard to *S. aureus*, it is well documented that this organism adheres to and invades cultured endothelial cell monolayers (Ogawa et al., 1985, *Infect. Immun.* 50: 218-224; Hamill et al., 1986, *Infect. and Imm.* 54:833-836) and that the cytotoxicity of ingested *S. aureus* is sensitive to the expression of known virulence factors (Vann and Proctor, 1988, *Micro. Patho.* 4:443-453). Such *ex vivo* models may afford more rapid and cost effective measurements of the efficacy of the "Off-Switch" experiments, and may be employed as preliminary analyses prior to testing in one or more of the animal models described above.

IX. Alternate Switch Designs and Control Mechanisms

As suggested above, a large variety of mechanisms can be used to control the level of activity of a product of a test gene, all within the scope of this invention. The design exemplified above utilizes transcriptional control based on the ability of a repressor molecule (*lac* repressor) to stop transcription from an endogenous promoter (P3 promoter). Expression of that repressor is then controlled by a second promoter (P_{blaZ}). Expression from that promoter is turned on by the release of the β-lactamase repressor in response to the presence of a β-lactam (CBAP) which does not have significant antibiotic activity. Therefore, this system requires the production of the blaR1 receptor molecule and the β-lactamase repressor.

However, genetic switches for use in the methods of this invention, which are conceptually quite similar, can be constructed using other regulatory components with similar functions to those described just above. In addition, the promoter controlling transcription of the test gene may be the promoter which normally controls the test gene, a different endogenous promoter, or a heterologous promoter. Some specific repressor/operator pairs are the following:

(1) *lexA* protein/*boxA* operator. This pair could replace the *lac* repressor/*lacO* operator. Production of the *lexA* protein can be controlled by the β-lactamase or other control system. (Kenyon et al., 1982.)

(2) *tet* repressor/*tet* operator. The *tet* system can clearly replace the β-lactamase system in the exemplified switch design.

(3) *trp* repressor/*trp* operator. The *trp* system can replace the β-lactamase system in the exemplified design. The *trp* repressor could be expressed constitutively, the addition of L-tryptophan activates the repressor causing it to bind to the operator site. Use of this system in this manner requires that the repressor is not significantly activated by tryptophan from the infection host.

(4) other negative regulatory element pairs listed in V. above.

While the above examples could be used as described, other such regulators are known to those skilled in the art, and the use of such regulators in the methods of this invention is within the scope of the invention and of the claims.

In addition to the design of a switch using two negative regulatory elements, other switch designs can be used to control transcription of a test gene. Among the possible designs are the following:

(1) Replace the β-lactamase repressor/operator system with a positive activator system, such as the *ara* system. The *ara* promoter would be transcriptionally linked with the *lacI* gene (coding for repressor) or a gene coding for another suitable repressor. This promoter is activated by *araC* protein in the presence of arabinose, so addition of arabinose would cause the production of the *lac* repressor, turning off expression of the test gene.

(2) Use of a repressor which is activated by a small molecule to turn off transcription of a test gene. Examples of such repressor molecules are the *arg* and *trp* repressors, which require the presence of arginine and tryptophan, respectively, to bind to their respective operators. In these switch designs, the repressor can be expressed constitutively since the repressor does not bind significantly to the operator in the absence of the appropriate repressor activator. Thus, if an operator which binds a repressor of this type is placed in *cis* with a test gene, addition of the appropriate small molecule will cause the repressor to bind to the operator, blocking transcription.

(3) Use of an "On" switch instead of an "Off" switch. In certain evaluations, turning a test gene on rather than off may be useful. Such an "On" switch can readily be constructed using either activation or derepression. For example, the *ara* operator described above could be linked in *cis* with a test gene and an appropriate promoter. If *araC* protein is also produced, the addition of arabinose will turn on expression of the test gene. (Raibaut & Schwartz, 1984, *Ann. Rev. Genet.* 18:415-444.) An example of derepression utilizes IPTG (isopropyl-β-D-thiogalactopyranoside) and the *lac* repressor/operator pair. With the *lac* repressor present and the *lac* operator in *cis* with the test gene, the *lac* repressor

will bind to the operator. This blocks expression of the test gene. Addition of IPTG inactivates the repressor, releasing it from the operator and allowing transcription of the test gene. (Note that derepression of *lac* with IPTG can also be used in the off switch design using two negative regulators. If the *lac* repressor/operator pair is used to control expression of a second repressor, the *lac* repressor is released by the addition of IPTG. This then allows expression of the second repressor, which then binds to its respective operator, blocking transcription of the test gene.)

(4) Use of antisense oligonucleotides (including hybrid polymers containing nucleic acid analogs) to block translation or transcription. As was mentioned above, antisense molecules complementary to a portion of an mRNA critical for translation can be introduced into a cell to block translation. Such molecules can be RNA oligonucleotides produced within the cell under the control of an artificially-controllable promoter. Alternatively, modified antisense polymers can be used which contain nucleic acid analogs, which are more resistant to degradation. Such molecules can be administered in a form which can be taken up by the infecting microbes, and which then will block expression of the test gene by binding to the corresponding mRNA. Antisense molecules can also be used to inhibit transcription. Complementary antisense molecules, under some conditions, will bind to the sense strand of the DNA to form triplex structures. Therefore, if the antisense sequence is selected to be complementary to an appropriate sense strand sequence, transcription is blocked when the antisense molecule is present. (Uhlmann & Peyman, 1990.)

(5) Use of ribozymes to block translation of test gene mRNA. Ribozymes can be provided by similar

approaches as discussed for antisense molecules. All-RNA ribozymes can be produced with microbial cells under artificial control, or modified ribozymes containing nucleic acid analogues can be administered in a form which will, as with antisense, be taken up by the microbial cells. With either approach, the ribozymes will then cut the mRNA corresponding to the test gene, preventing (or at least significantly reducing) expression of the test gene. (Eckstein et al., 1992.)

In addition to the control designs and mechanisms described above, any mechanism which provides specific artificial control of the level of activity of a product of a test gene is potentially useful in the methods of this invention, and is included within the scope of this invention.

X. Eukaryotic Microbes and Test Genes

While the molecular biology of prokaryotes is, in general, better understood than that of eukaryotes, the use of eukaryotic microbial pathogens and test genes is within the scope of this invention. Those skilled in the field will recognize that there are also features of eukaryotic biology, differing from prokaryotic biology, which must be addressed in the practice of this invention with eukaryotic pathogens. These additional concerns include at least the following: (a) In diploid organisms, two copies of a gene are present. In many cases both copies must be inactivated to allow evaluation of an artificially-controllable copy of a corresponding test gene; (b) Control mechanisms, especially transcriptional control mechanisms, are often more complex, involving multiple components; (c) Chromosome structure is often more complex and more important to gene expression; (d) Recombinant techniques are often more difficult, due to such factors are the large size of eukaryotic chromosomes;

(e) Gene expression often involves additional processing steps, such as mRNA splicing.

However, those skilled in the field recognize that there are control systems, even in higher eukaryotes which can provide appropriate control for use in the methods of this invention. For example, the steroid and thyroid hormones (*e.g.*, cortisol, steroid sex hormones, and ecdysone) and retinoids are widespread, and the control systems responding to those hormones regulate the transcription of specific genes. Thus, such control can be used to control specific test genes.

In addition, in the lower eukaryotes, a variety of control systems are known which can be used in this invention. An example is the system responding to the mating factor peptide from *Saccharomyces cerevisiae*. Further, a variety of recombinant techniques are available for manipulating nucleic acid sequences in lower eukaryotic microbes. For instance, the genes required for the components of a switch can, for yeasts, be incorporated into a YAC (yeast artificial chromosome); similar mini-chromosomes can be constructed for some other eukaryotic microbes as well.

XI. Viral Pathogens

In addition to the use of the methods of this invention to evaluate genes of pathogenic microbes which are themselves cells, these methods are also applicable to viruses and viral genes. While viruses utilize the host cellular machinery to express the viral-encoded genes, at least the products of those genes are potential therapeutic targets. Also, for many viral pathogens, the functions of those viral-encoded genes are not known. Therefore, this invention is especially useful for evaluating the viral-encoded genes as potential targets by determining the effects of turning off the activity of the viral-encoded gene products after infection has been established.

In general, it is necessary to package the artificially-controllable copy of the gene in the viral structure by inserting that copy in the viral genome. In some cases it is possible to inactivate the native copy and insert an additional gene sequence containing the artificially-controllable copy with the appropriate control component(s), such as an operator site in *cis*. In other cases it is necessary to replace the native copy with the artificially-controllable copy, such as when the amount of DNA which can be packaged in the virus is limited. Therefore, it is preferable that other components of the control system, not encoded by the host cells, be minimized.

Alternatively, if a cell-based infection model is used, the test gene and the required regulatory components can be inserted in a plasmid and the plasmid inserted in the cells used for the infection model, or inserted in a chromosome of the host cell using a transposon, other eukaryotic integration vector, or by random insertion.

It is also possible, in some cases, to insert foreign genes in the germ line of an organism (*e.g.*, a mouse), producing a transgenic animal. This technique can be used to produce an infection model host organism line which produces the molecules needed for controlling the expression of a viral gene. This obviates the packaging problems which can be caused by increasing the size of the viral genome.

The methods of this invention are applicable to both DNA and RNA viruses. In either case, an artificially controllable test gene can be inserted.

The embodiments described herein are not meant to be limiting to the invention. Those skilled in the art will appreciate that the invention may be practiced by using numerous microbial strains, species, and genes, as well as different designs of the DNA constructs, different selections of regulatory sequences and switching compounds, different mechanisms for control of the level

of activity, and various infection models for *in vivo* evaluation, all within the breadth of the claims.

Claims

1. A method for evaluating a putative pathogenesis gene or essential gene as a target for antimicrobial treatment, comprising the steps of:

(a) infecting a mammal with a microbe containing an artificially-created genetic alteration, wherein said genetic alteration enables a change in the level of activity of a product of the coding sequence of said putative pathogenesis gene or essential gene in said microbe in response to an artificial change in the environment of said microbe,

(b) changing the environment of said microbe in a manner which will cause a change in said level of activity of said product of said coding sequence,

(c) determining whether the state of the infection or the physiological condition of said mammal is altered in response to said change in said level of activity of a product of said coding sequence,

wherein said putative pathogenesis gene or essential gene is a target if the state of the infection or the physiological condition of the mammal is altered in response to said change in said level of activity of a product of said coding sequence.

2. The method of claim 1, wherein said change in the environment of said microbe comprises contacting said microbe with a switching compound.

3. The method of claim 2, further comprising a plurality of said artificially-created genetic alterations,

wherein each said artificially-created genetic alteration enables a change in the level of activity of a product of a coding sequence of said putative pathogenesis gene or essential gene, and

wherein at least two of said artificially-created genetic alterations enable a change in said level of activity in response to different switching compounds.

4. The method of claim 2, further comprising a plurality of said artificially-created genetic alterations and a plurality of said putative pathogenesis genes or essential genes,

wherein each said artificially-created genetic alteration enables a change in the level of activity of a product of a coding sequence of a said putative pathogenesis gene or essential gene,

wherein at least two of said artificially-created genetic alterations enable a change in said level of activity in response to different switching compounds, and

wherein the levels of activity of the products of the coding sequences of at least two of said putative pathogenesis genes or essential genes are changed in response to different said switching compounds.

5. A method for evaluating a putative pathogenesis gene or essential gene as a target for antimicrobial treatment, comprising the steps of:

(a) infecting a mammal with a recombinant microbe, wherein said recombinant microbe contains at least one DNA construct comprising a putative pathogenesis gene or essential gene, and wherein the expression state of said gene can be switched by contacting said microbe with a switching compound;

(b) contacting said recombinant microbe with said switching compound; and

(c) determining whether the state of the infection or the physiological condition of said mammal is altered in response to the switch of the expression state of said putative pathogenesis gene or essential gene;

wherein said putative pathogenesis gene or essential gene is a target if the state of the infection or the physiological condition of the mammal is altered in response to said switch of the expression state of said putative pathogenesis gene or essential gene.

6. The method of claim 5, wherein said recombinant microbe is a recombinant bacterium.

7. The method of claim 6, wherein said recombinant bacterium is a Staphylococcus species.

8. The method of claim 7, wherein said Staphylococcus species is *Staphylococcus aureus*.

9. The method of claim 8, wherein said putative pathogenesis gene or essential gene is a pathogenesis gene.

10. The method of claim 9, wherein said putative pathogenesis gene is from the *agr* locus.

11. The method of claim 6, wherein said putative pathogenesis gene or essential gene is an essential gene.

12. The method of claim 6, wherein said recombinant bacterium is a Pseudomonas species.

13. The method of claim 5, wherein said recombinant microbe is a virus.

14. The method of claim 5, wherein said recombinant microbe is a lower eukaryote.

15. The method of claim 5, wherein said recombinant microbe is a yeast.

16. The method of claim 5, wherein said recombinant microbe is a fungus.

17. The method of claim 5, wherein said recombinant microbe is a protozoan.

18. The method of claim 5, wherein said putative pathogenesis gene or essential gene is derived from a different microbial species than said microbe.

19. The method of claim 6 wherein said putative pathogenesis gene or essential gene is derived from a different bacterial species than said recombinant bacterium.

20. The method of claim 6 wherein said putative pathogenesis gene is derived from a yeast or lower eukaryote.

21. The method of claim 5 or 6, wherein said DNA construct further comprises a chromosomally-inserted, artificially controllable promoter.

22. The method of claim 21, wherein said chromosomally-inserted, artificially controllable promoter is transcriptionally linked with said putative pathogenesis gene or essential gene.

23. A method for evaluating a putative pathogenesis gene or essential gene as a target for antimicrobial treatment, comprising the steps of:

(a) infecting a plant with a microbe containing an artificially-created genetic alteration, wherein said genetic alteration enables a change in the level of activity of a product of a coding sequence in said microbe in response to a change in the environment of said microbe,

(b) changing the environment of said microbe in a manner which will cause said change in said level of activity of a product of a coding sequence,

(c) determining whether the state of the infection or the physiological condition of said plant is altered in response to said change is said level of activity of a product of a coding sequence,

wherein said putative pathogenesis gene or essential gene is a target if the state of the infection or the physiological condition of the plant is altered in response to said change in said level of activity of a product of a coding sequence.

24. The method of claim 23, wherein said change in the environment of said microbe comprises contacting said microbe with a switching compound.

25. The method of claim 24, wherein said microbe is a fungus.

26. A recombinant microbial strain expressing a repressor molecule which enables artificial control of the level of activity of the product of a test gene and having an artificially-inserted DNA construct comprising an operator site to which said repressor molecule can bind,

wherein said operator site is linked with a test gene, and

wherein binding of said repressor molecule to said operator site blocks expression of said test gene.

27. The recombinant microbial strain of claim 26, wherein said DNA construct is chromosomally-inserted.

28. The recombinant microbial strain of claim 26, wherein said microbial strain is a bacterial strain.

29. The recombinant bacterial strain of claim 28, wherein said strain is derived from a *Staphylococcus* species.

30. The recombinant bacterial strain of claim 29, which is a strain of *Staphylococcus aureus*.

31. The recombinant bacterial strain of claim 28 or 30, which expresses repressor molecules for a *lac* regulatory system and a *bla* regulatory system, wherein expression of the *lac* repressor molecule is controlled by the *bla* promoter.

32. The strain of claim 31, comprising *lacO*, *lacI* transcriptionally linked with P_{blaZ}, and *blaI* and *blaR1* transcriptionally linked with P_{blaR1}.

33. The strain of claim 32, containing a DNA construct comprising the P3 promoter transcriptionally linked with the *hld*/RNAIII gene, and

further comprising a *lacO* site,

wherein binding of a lac repressor molecule to said *lacO* site blocks expression of said *hld*/RNAIII gene.

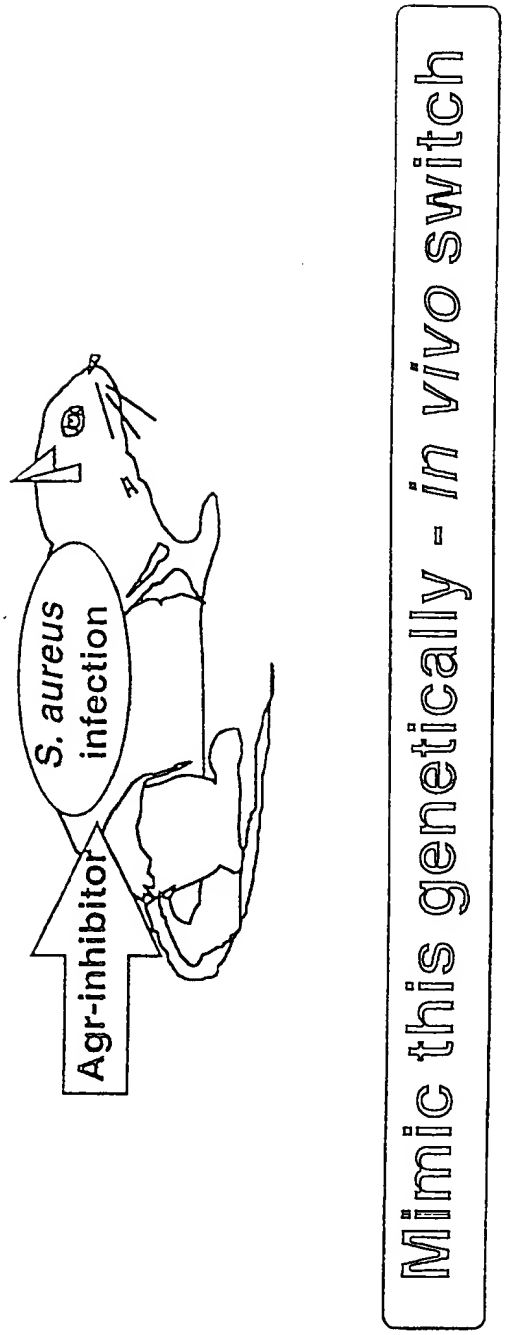

Mimic this genetically - in vivo switch
S. aureus infection
Agr-inhibitor

FIG. 1.

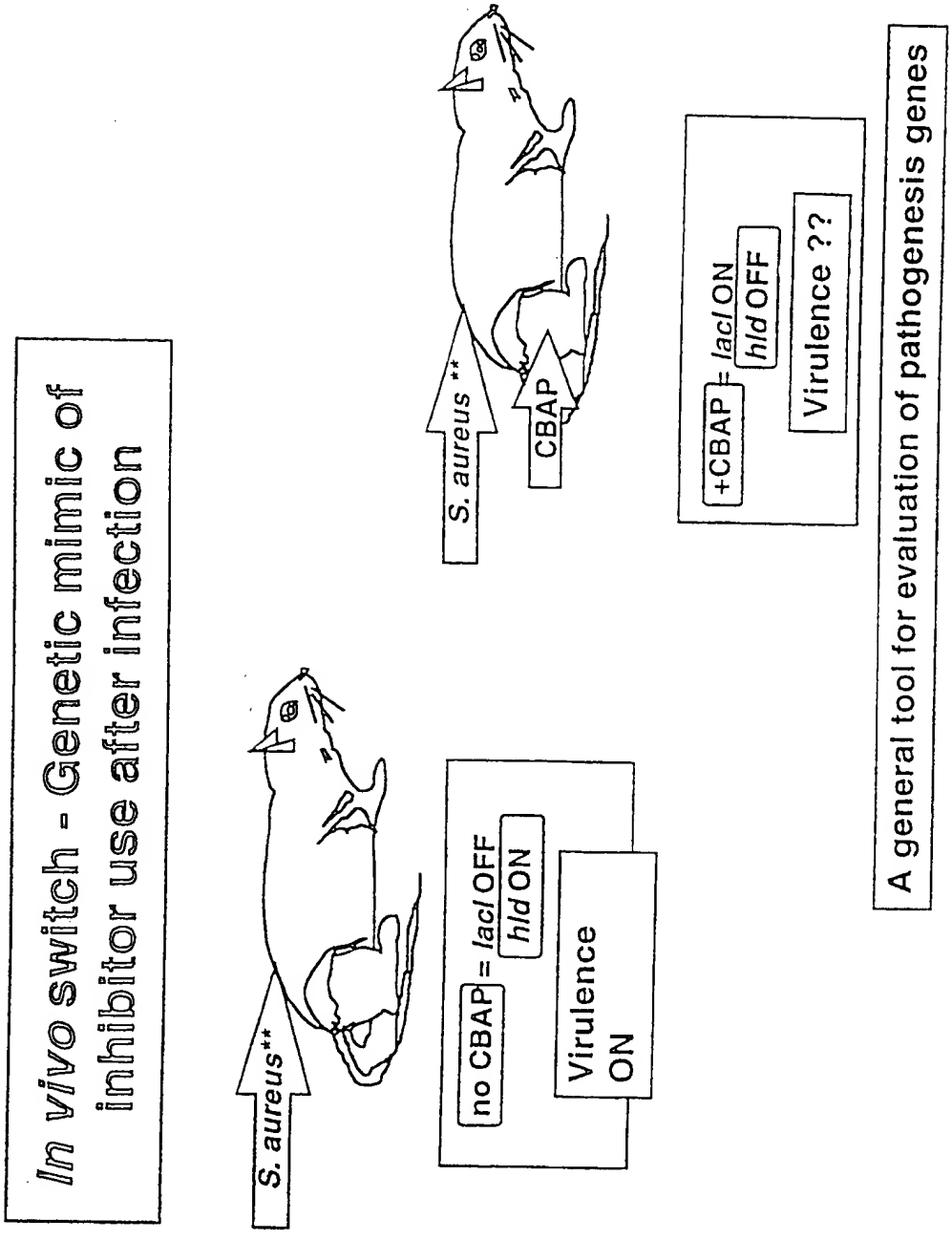
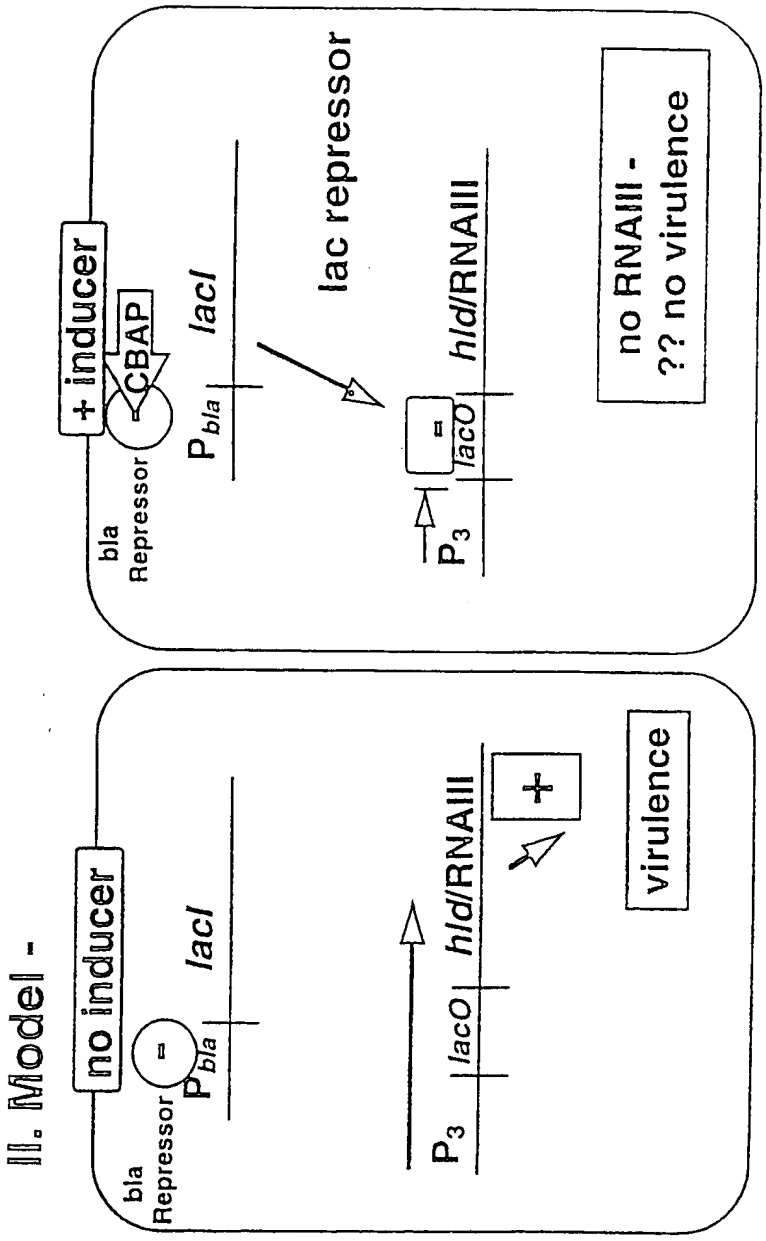

In vivo switch - Genetic mimic of inhibitor use after infection
S. aureus**
no CBAP = lacI OFF hld ON
Virulence ON
S. aureus **
CBAP
+CBAP = lacI ON hld OFF
Virulence ??
A general tool for evaluation of pathogenesis genes

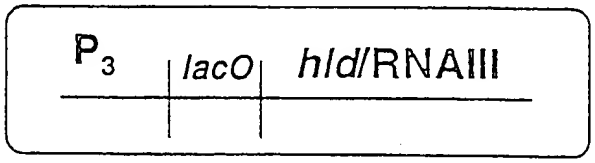
FIG. 2.

FIG. 3.


II. Model -
no inducer
bla Repressor
P bla
lacI
P3
lacO
hld/RNAIII
virulence
+ inducer
CBAP
lac repressor
no RNAIII -
?? no virulence

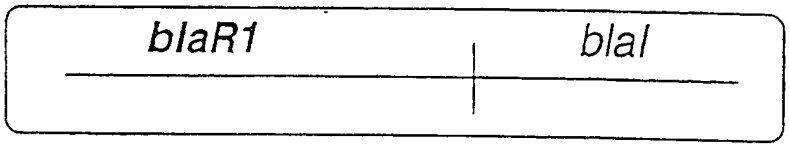
I. Plasmid constructs -

1. Modified P3-RNAIII allele:



2. Inducible lac repressor:

a.



b.

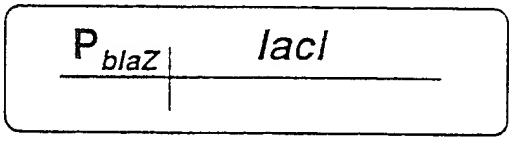
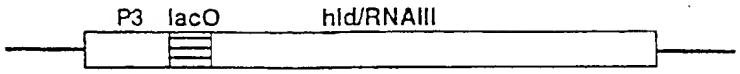


FIG. 4.

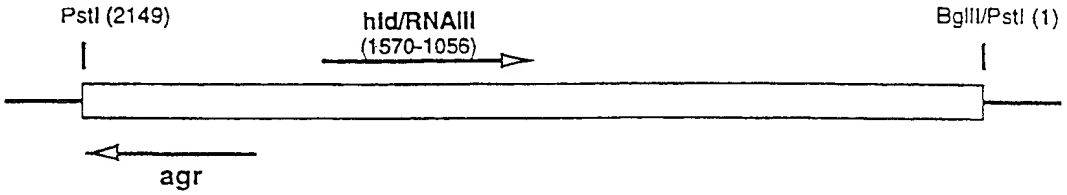
FIG. 5.

P3-lacO-RNAIII

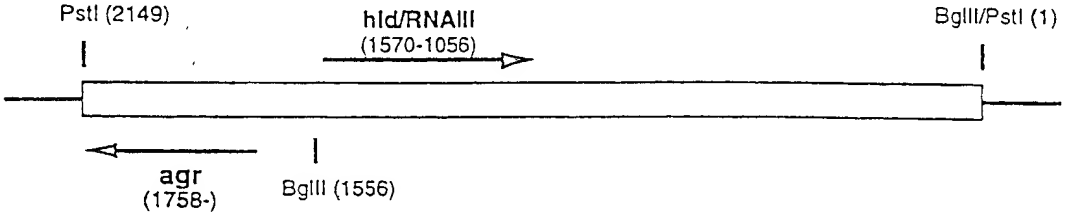


Construction method

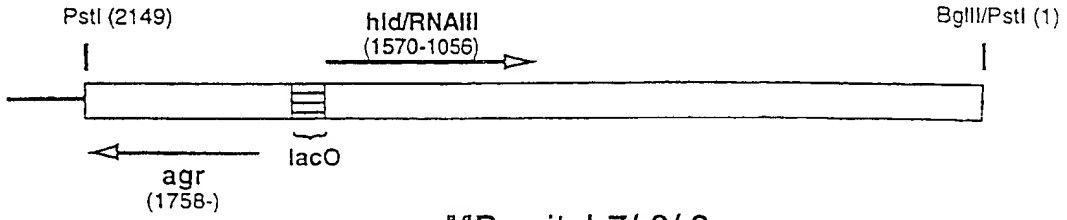
1. SUBCLONE 2149 bp PstI-Bg1II AGR-hld/RNAIII-CONTAINING GENOMIC FRAGMENT FROM PLASMID pEX07 (JANZON AND ARVIDSON 1990) INTO PstI SITE OF PLASMID pALTER-1 (PROMEGA) FOR IN VITRO SITE-DIRECTED MUTAGENESIS.



2. CREATE UNIQUE Bg1II SITE IN P3 (C1557 TO G) WITH MUTAGENIC OLIGONUCLEOTIDE PIVS5, CREATING PLASMID pMP153



3. INSERT 21 bp lacO OLIGONUCLEOTIDE AT Bg1II 1556 OF P3 (3 CONFIGURATIONS: 1 OLIGO, FORWARD ORIENTATION; 1 OLIGO REVERSE ORIENTATION; 3 TANDEM OLIGOS, FORWARD ORIENTATION)

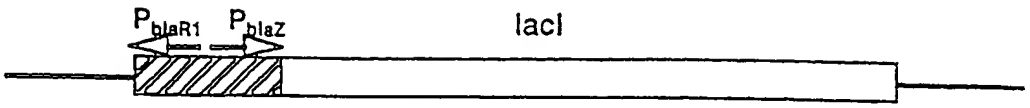


pMPswitch7/ 8/ 9

4. SUBCLONE P3-lacO-RNAIII PstI FRAGMENT FROM (3) TO *S. aureus-E. coli*. SHUTTLE PLASMID pMP157, CREATING PLASMIDS pMPswitch 7, 8, 9.

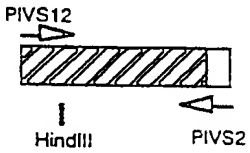
FIG. 6.

P_{blaZ}-lacI

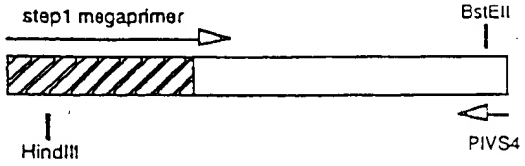


Construction method

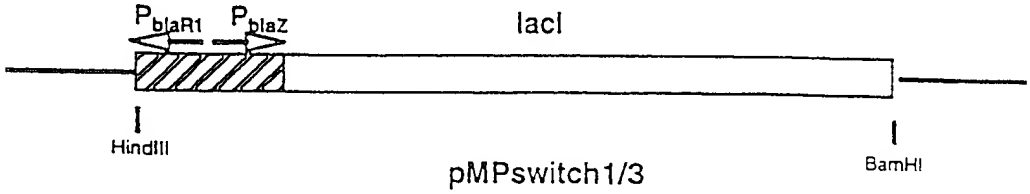
1. PCR AMPLIFY 242 bp *blaR1-blaZ* PROMOTER-CONTAINING FRAGMENT (pI524 AS TEMPLATE) WITH 3' COMPLEMENTARITY TO *E. coli. lacI*



2. FUSE *blaZ* PROMOTER TO 5' PORTION of *lacI* CODING SEQUENCE BY PCR USING
PRODUCT OF STEP 1 AS MEGAPRIMER



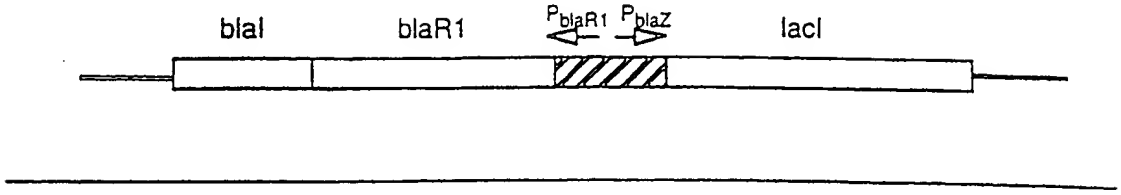
3. RESTRICT PRODUCT OF STEP 2 (HindIII + BstEII), LIGATE WITH BstEII-BamHI *lacI* 3' FRGMENT (FROM pDG148) AND HindIII-BamHI pBLUESCRIPT Ks+ (STRATAGENE)



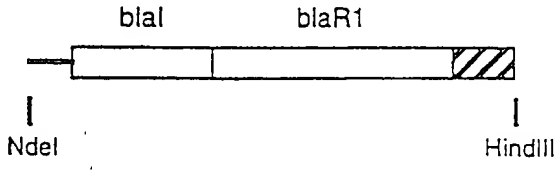
4. SUBCLONE HindIII-BamHIP_{blaZ} -*lacI* FROM pMPswitch 1 TO *S. aureus-E. coli* SHUTTLE PLASMID pMP16, CREATING PLASMID pMPswitch3.

FIG. 7.

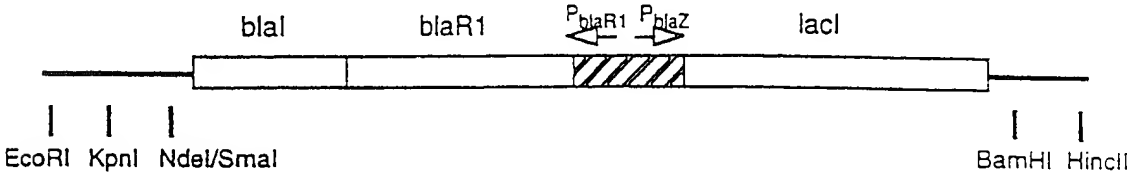
blaI-blaR1-P_{blaZ}-lacI



1. RESTRICT pCH1712 WITH HindIII AND NdeI, BLUNT NdeI SITE, GEL ISOLATE *blaR1-blaI*FRAGMENT



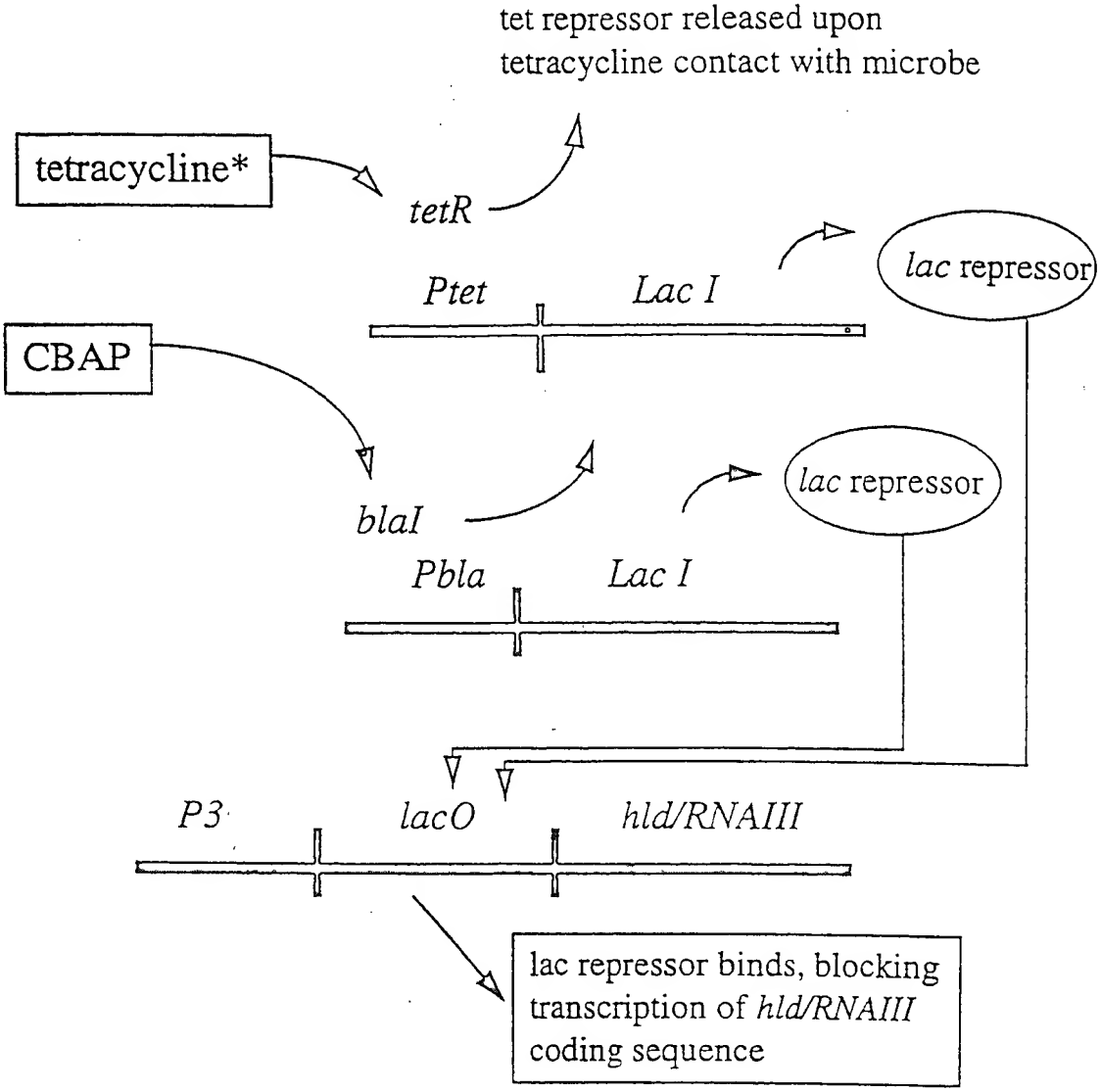
2. RESTRICT pMPswitch3 WITH HindIII AND SmaI, LIGATE WITH *blaR1-blaI* FROM STEP 1



pMPswitch4

3. SUBCLONE *blaI-blaR1-*P_{blaZ} *-lacI* FROM pMPswitch4 TO *S. aureus* INTEGRATION VECTOR pXX, CREATING PLASMID pMPswitch5.

FIG. 8.



Recombinant *Staphylococcus aureus* cells having two constructs able to produce lac repressor. The first produces lac repressor when the cell is contacted with tetracycline; the second when the cell is contacted with a beta-lactam *e.g.* CBAP.

*IN THE CASE OF TETRACYCLINE, THE SMALL MOLECULE INDUCER (TETRACYCLINE) NOT ONLY "CONTACTS" BUT DIFFUSES INTO THE TARGET MICROBE.

INTERNATIONAL SEARCH REPORT

International Application No
US 96/07937

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12Q1/02 C12N15/63

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12Q C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	J. BACTERIOL. (1991), 173(20), 6313-20 CODEN: JOBAAY;ISSN: 0021-9193, 1991, XP002017658 VANDENESCH, FRANCOIS ET AL: "A temporal signal, independent of agr, is required for hla but not spa transcription in Staphylococcus aureus" see the whole document	1,2, 5-10, 26-33
X	US,A,5 362 646 (BUJARD HERMANN ET AL) 8 November 1994 see column 6, line 39 - line 57; claims 9,10	26-33
P,X	WO,A,96 10579 (UNIV NEW YORK) 11 April 1996 see page 13, line 26 - page 14, line 6	1,5-10

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
13 November 1996	26 -11- 1996

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Hoekstra, S

Form PCT/ISA/210 (second sheet) (July 1992)

International Application No
PCT/US 96/07937

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO,A,90 15143 (AGRONOMIQUE INST NAT RECH ;CENTRE NAT RECH SCIENT (FR)) 13 December 1990 see the whole document	1-25
A	J. INFECT. DIS. (1995), VOLUME DATE 1995, 172(2), 410-19 CODEN: JIDIAQ;ISSN: 0022-1899, 1995, XP002017659 KERNODLE, DOUGLAS S. ET AL: "Growth of Staphylococcus aureus with nafcillin in vitro induces.alpha.-toxin production and increases the lethal activity of sterile broth filtrates in a murine model" see the whole document	1-25

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 96/07937

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
US-A-5362646	08-11-94	AT-T-	123526	15-06-95
		DE-D-	3853932	13-07-95
		EP-A-	0303925	22-02-89
		JP-A-	1095790	13-04-89
WO-A-9610579	11-04-96	AU-A-	3825995	26-04-96
WO-A-9015143	13-12-90	FR-A-	2648149	14-12-90
		AP-A-	148	22-10-91
		AU-A-	5839990	07-01-91
		EP-A-	0476025	25-03-92